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(54) Title: PRODUCTION OF GAMMA LINOLENIC ACID BY A $\Delta 6$ -DESATURASE (57) Abstract <p>Linoleic acid is converted into γ-linolenic acid by the enzyme $\Delta 6$-desaturase. The present invention is directed to isolated nucleic acids comprising the $\Delta 6$-desaturase gene. More particularly, the isolated nucleic acid comprises the promoter, coding region and termination regions of the $\Delta 6$-desaturase gene. The present invention provides recombinant constructions comprising the $\Delta 6$-desaturase coding region in functional combination with heterologous regulatory sequences. The nucleic acids and recombinant constructions of the instant invention are useful in the production of GLA in transgenic organisms.</p>		

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1 PRODUCTION OF GAMMA LINOLENIC ACID BY A $\Delta 6$ -DESATURASE

Linoleic acid (18:2) (LA) is transformed into gamma linolenic acid (18:3) (GLA) by the enzyme
5 $\Delta 6$ -desaturase. When this enzyme, or the nucleic acid encoding it, is transferred into LA-producing cells, GLA is produced. The present invention provides nucleic acids comprising the $\Delta 6$ -desaturase gene. More specifically, the nucleic acids comprise the
10 promoters, coding regions and termination regions of the $\Delta 6$ -desaturase genes. The present invention is further directed to recombinant constructions comprising a $\Delta 6$ -desaturase coding region in functional combination with heterologous regulatory sequences.
15 The nucleic acids and recombinant constructions of the instant invention are useful in the production of GLA in transgenic organisms.

Unsaturated fatty acids such as linoleic ($C_{18}\Delta^{9,12}$) and α -linolenic ($C_{18}\Delta^{9,12,15}$) acids are essential
20 dietary constituents that cannot be synthesized by vertebrates since vertebrate cells can introduce double bonds at the Δ^9 position of fatty acids but cannot introduce additional double bonds between the Δ^9 double bond and the methyl-terminus of the fatty
25 acid chain. Because they are precursors of other products, linoleic and α -linolenic acids are essential fatty acids, and are usually obtained from plant sources. Linoleic acid can be converted by mammals into γ -linolenic acid (GLA, $C_{18}\Delta^{6,9,12}$) which can in turn
30 be converted to arachidonic acid (20:4), a critically

1 important fatty acid since it is an essential
precursor of most prostaglandins.

The dietary provision of linoleic acid, by
virtue of its resulting conversion to GLA and
5 arachidonic acid, satisfies the dietary need for GLA
and arachidonic acid. However, a relationship has
been demonstrated between consumption of saturated
fats and health risks such as hypercholesterolemia,
atherosclerosis and other clinical disorders which
10 correlate with susceptibility to coronary disease,
while the consumption of unsaturated fats has been
associated with decreased blood cholesterol
concentration and reduced risk of atherosclerosis.
The therapeutic benefits of dietary GLA may result
15 from GLA being a precursor to arachidonic acid and
thus subsequently contributing to prostaglandin
synthesis. Accordingly, consumption of the more
unsaturated GLA, rather than linoleic acid, has
potential health benefits. However, GLA is not
20 present in virtually any commercially grown crop
plant.

Linoleic acid is converted into GLA by the
enzyme $\Delta 6$ -desaturase. $\Delta 6$ -desaturase, an enzyme of
more than 350 amino acids, has a membrane-bound domain
25 and an active site for desaturation of fatty acids.
When this enzyme is transferred into cells which
endogenously produce linoleic acid but not GLA, GLA is
produced. The present invention, by providing the
gene encoding $\Delta 6$ -desaturase, allows the production of
30 transgenic organisms which contain functional $\Delta 6$ -
desaturase and which produce GLA. In addition to

1 allowing production of large amounts of GLA, the
present invention provides new dietary sources of GLA.

The present invention is directed to
isolated $\Delta 6$ -desaturase genes. Specifically, the
5 isolated genes comprises the $\Delta 6$ -desaturase promoters,
coding regions, and termination regions.

The present invention is further directed to
expression vectors comprising the $\Delta 6$ -desaturase
promoter, coding region and termination region.

10 Yet another aspect of this invention is
directed to expression vectors comprising a $\Delta 6$ -
desaturase coding region in functional combination
with heterologous regulatory regions, i.e. elements
not derived from the $\Delta 6$ -desaturase gene.

15 Cells and organisms comprising the vectors
of the present invention, and progeny of such
organisms, are also provided by the present invention.

A further aspect of the present invention
provides isolated bacterial $\Delta 6$ -desaturase. An
20 isolated plant $\Delta 6$ -desaturase is also provided.

Yet another aspect of this invention
provides a method for producing plants with increased
gamma linolenic acid content.

A method for producing chilling tolerant
25 plants is also provided by the present invention.

Fig. 1 depicts the hydropathy profiles of
the deduced amino acid sequences of Synechocystis $\Delta 6$ -
desaturase (Panel A) and $\Delta 12$ -desaturase (Panel B).
Putative membrane spanning regions are indicated by
30 solid bars. Hydrophobic index was calculated for a

1 window size of 19 amino acid residues [Kyte, et al.
(1982) J. Molec. Biol. 157].

Fig. 2 provides gas liquid chromatography
profiles of wild type (Panel A) and transgenic (Panel
5 B) Anabaena.

Fig. 3 is a diagram of maps of cosmid cSy75,
cSy13 and Csy7 with overlapping regions and subclones.
The origins of subclones of Csy75, Csy75-3.5 and Csy7
are indicated by the dashed diagonal lines.
10 Restriction sites that have been inactivated are in
parentheses.

Fig. 4 provides gas liquid chromatography
profiles of wild type (Panel A) and transgenic (Panel
B) tobacco.

15 Fig. 5A depicts the DNA sequence of a Δ -6
desaturase cDNA isolated from borage.

Fig. 5B depicts the protein sequence of the
open reading frame in the isolated borage Δ -6
desaturase cDNA. Three amino acid motifs
20 characteristic of desaturases are indicated and are,
in order, lipid box, metal box 1, and metal box 2.

Fig. 6 is a dendrogram showing similarity of
the borage Δ 6-desaturase to other membrane-bound
desaturases. The amino acid sequence of the borage
25 Δ 6-desaturase was compared to other known desaturases
using Gene Works (IntelliGenetics). Numerical values
correlate to relative phylogenetic distances between
subgroups compared.

Fig. 7 is a restriction map of 221. Δ 6.NOS
30 and 121. Δ 6.NOS. In 221. Δ 6.NOS, the remaining portion

1 of the plasmid is pBI221 and in 121.Δ6.NOS, the
remaining portion of the plasmid is pBI121.

Fig. 8 provides gas liquid chromatography
profiles of mock transfected (Panel A) and 221.Δ6.NOS
5 transfected (Panel B) carrot cells. The positions of
18:2, 18:3 α, and 18:3 γ (GLA) are indicated.

Fig. 9 provides gas liquid chromatography
profiles of an untransformed tobacco leaf (Panel A)
and a tobacco leaf transformed with 121.Δ6.NOS. The
10 positions of 18:2, 18:3 α, 18:3 γ (GLA), and 18:4 are
indicated.

Fig. 10 provides gas liquid chromatography
profiles for untransformed tobacco seeds (Panel A) and
seeds of tobacco transformed with 121.Δ6.NOS. The
15 positions of 18:2, 18:3α and 18:3γ (GLA) are indicated.

The present invention provides isolated
nucleic acids encoding Δ6-desaturase. To identify a
nucleic acid encoding Δ6-desaturase, DNA is isolated
from an organism which produces GLA. Said organism
20 can be, for example, an animal cell, certain fungi
(e.g. Mortierella), certain bacteria (e.g.
Synechocystis) or certain plants (borage, Oenothera,
currants). The isolation of genomic DNA can be
accomplished by a variety of methods well-known to one
25 of ordinary skill in the art, as exemplified by
Sambrook et al. (1989) in Molecular Cloning: A
Laboratory Manual, Cold Spring Harbor, NY. The
isolated DNA is fragmented by physical methods or
enzymatic digestion and cloned into an appropriate
30 vector, e.g. a bacteriophage or cosmid vector, by any
of a variety of well-known methods which can be found

1 in references such as Sambrook et al. (1989).
Expression vectors containing the DNA of the present
invention are specifically contemplated herein. DNA
encoding $\Delta 6$ -desaturase can be identified by gain of
5 function analysis. The vector containing fragmented
DNA is transferred, for example by infection,
transconjugation, transfection, into a host organism
that produces linoleic acid but not GLA. As used
herein, "transformation" refers generally to the
10 incorporation of foreign DNA into a host cell.
Methods for introducing recombinant DNA into a host
organism are known to one of ordinary skill in the art
and can be found, for example, in Sambrook et al.
(1989). Production of GLA by these organisms (i.e.,
15 gain of function) is assayed, for example by gas
chromatography or other methods known to the
ordinarily skilled artisan. Organisms which are
induced to produce GLA, i.e. have gained function by
the introduction of the vector, are identified as
20 expressing DNA encoding $\Delta 6$ -desaturase, and said DNA is
recovered from the organisms. The recovered DNA can
again be fragmented, cloned with expression vectors,
and functionally assessed by the above procedures to
define with more particularity the DNA encoding $\Delta 6$ -
25 desaturase.

As an example of the present invention,
random DNA is isolated from the cyanobacteria
Synechocystis Pasteur Culture Collection (PCC) 6803,
American Type Culture Collection (ATCC) 27184, cloned
30 into a cosmid vector, and introduced by
transconjugation into the GLA-deficient cyanobacterium

1 Anabaena strain PCC 7120, ATCC 27893. Production of GLA from Anabaena linoleic acid is monitored by gas chromatography and the corresponding DNA fragment is isolated.

5 The isolated DNA is sequenced by methods well-known to one of ordinary skill in the art as found, for example, in Sambrook et al. (1989).

In accordance with the present invention, DNA molecules comprising $\Delta 6$ -desaturase genes have been
10 isolated. More particularly, a 3.588 kilobase (kb) DNA comprising a $\Delta 6$ -desaturase gene has been isolated from the cyanobacteria Synechocystis. The nucleotide sequence of the 3.588 kb DNA was determined and is shown in SEQ ID NO:1. Open reading frames defining
15 potential coding regions are present from nucleotide 317 to 1507 and from nucleotide 2002 to 3081. To define the nucleotides responsible for encoding $\Delta 6$ -desaturase, the 3.588 kb fragment that confers $\Delta 6$ -desaturase activity is cleaved into two subfragments,
20 each of which contains only one open reading frame. Fragment ORF1 contains nucleotides 1 through 1704, while fragment ORF2 contains nucleotides 1705 through 3588. Each fragment is subcloned in both forward and reverse orientations into a conjugal expression vector
25 (AM542, Wolk et al. [1984] Proc. Natl. Acad. Sci. USA 81, 1561) that contains a cyanobacterial carboxylase promoter. The resulting constructs (i.e. ORF1(F), ORF1(R), ORF2(F) and ORF2(R)) are conjugated to wild-type Anabaena PCC 7120 by standard methods (see, for
30 example, Wolk et al. (1984) Proc. Natl. Acad. Sci. USA 81, 1561). Conjugated cells of Anabaena are

1 identified as Neo^r green colonies on a brown
background of dying non-conjugated cells after two
weeks of growth on selective media (standard mineral
media BG11N + containing 30µg/ml of neomycin according
5 to Rippka et al., (1979) J. Gen Microbiol. 111, 1).
The green colonies are selected and grown in selective
liquid media (BG11N + with 15µg/ml neomycin). Lipids
are extracted by standard methods (e.g. Dahmer et al.,
(1989) Journal of American Oil Chemical Society 66,
10 543) from the resulting transconjugants containing the
forward and reverse oriented ORF1 and ORF2 constructs.
For comparison, lipids are also extracted from wild-
type cultures of Anabaena and Synechocystis. The
fatty acid methyl esters are analyzed by gas liquid
15 chromatography (GLC), for example with a Tracor-560
gas liquid chromatograph equipped with a hydrogen
flame ionization detector and a capillary column. The
results of GLC analysis are shown in Table 1.

20

25

30

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1 Table 1: Occurrence of C18 fatty acids in wild-type
and transgenic cyanobacteria

5	SOURCE	18:0	18:1	18:2	γ 18:3	α 18:3	18:4
	Anabaena (wild type)	+	+	+	-	+	-
	Anabaena + ORF1(F)	+	+	+	-	+	-
	Anabaena + ORF1(R)	+	+	+	-	+	-
	Anabaena + ORF2(F)	+	+	+	+	+	+
10	Anabaena + ORF2(R)	+	+	+	-	+	-
	Synechocystis (wild type)	+	+	+	+	-	-

15 As assessed by GLC analysis, GLA deficient
Anabaena gain the function of GLA production when the
construct containing ORF2 in forward orientation is
introduced by transconjugation. Transconjugants
containing constructs with ORF2 in reverse orientation
to the carboxylase promoter, or ORF1 in either
20 orientation, show no GLA production. This analysis
demonstrates that the single open reading frame (ORF2)
within the 1884 bp fragment encodes Δ 6-desaturase.
The 1884 bp fragment is shown as SEQ ID NO:3. This is
substantiated by the overall similarity of the
25 hydropathy profiles between Δ 6-desaturase and Δ 12-
desaturase [Wada et al. (1990) Nature 347] as shown in
Fig. 1 as (A) and (B), respectively.

Also in accordance with the present
invention, a cDNA comprising a Δ 6-desaturase gene from
borage (Borago officinalis) has been isolated. The
30 nucleotide sequence of the 1.685 kilobase (kb) cDNA

1 was determined and is shown in Fig. 5A (SEQ ID NO: 4).
The ATG start codon and stop codon are underlined.
The amino acid sequence corresponding to the open
reading frame in the borage delta 6-desaturase is
5 shown in Fig. 5B (SEQ ID NO: 5).

Isolated nucleic acids encoding $\Delta 6$ -
desaturase can be identified from other GLA-producing
organisms by the gain of function analysis described
above, or by nucleic acid hybridization techniques
10 using the isolated nucleic acid which encodes
Synechocystis or borage $\Delta 6$ -desaturase as a
hybridization probe. Both genomic and cDNA cloning
methods are known to the skilled artisan and are
contemplated by the present invention. The
15 hybridization probe can comprise the entire DNA
sequence disclosed as SEQ. ID NO:1 or SEQ. ID NO:4, or
a restriction fragment or other DNA fragment thereof,
including an oligonucleotide probe. Methods for
cloning homologous genes by cross-hybridization are
20 known to the ordinarily skilled artisan and can be
found, for example, in Sambrook (1989) and Beltz et
al. (1983) Methods in Enzymology 100, 266.

In another method of identifying a delta 6-
desaturase gene from an organism producing GLA, a cDNA
25 library is made from poly-A⁺ RNA isolated from
polysomal RNA. In order to eliminate hyper-abundant
expressed genes from the cDNA population, cDNAs or
fragments thereof corresponding to hyper-abundant
cDNAs genes are used as hybridization probes to the
30 cDNA library. Non hybridizing plaques are excised and
the resulting bacterial colonies are used to inoculate

1 liquid cultures and sequenced. For example, as a
means of eliminating other seed storage protein cDNAs
from a cDNA library made from borage polysomal RNA,
cDNAs corresponding to abundantly expressed seed
5 storage proteins are first hybridized to the cDNA
library. The "subtracted" DNA library is then used to
generate expressed sequence tags (ESTs) and such tags
are used to scan a data base such as GenBank to
identify potential desaturates.

10 Transgenic organisms which gain the function
of GLA production by introduction of DNA encoding Δ -
desaturase also gain the function of
octadecatetraenoic acid ($18:4^{\Delta 6,9,12,15}$) production.
Octadecatetraenoic acid is present normally in fish
15 oils and in some plant species of the Boraginaceae
family (Craig et al. [1964] J. Amer. Oil Chem. Soc.
41, 209-211; Gross et al. [1976] Can. J. Plant Sci.
56, 659-664). In the transgenic organisms of the
present invention, octadecatetraenoic acid results
20 from further desaturation of α -linolenic acid by $\Delta 6$ -
desaturase or desaturation of GLA by $\Delta 15$ -desaturase.

The 359 amino acids encoded by ORF2, i.e.
the open reading frame encoding Synechocystis $\Delta 6$ -
desaturase, are shown as SEQ. ID NO:2. The open
25 reading frame encoding the borage $\Delta 6$ -desaturase is
shown in SEQ ID NO: 5. The present invention further
contemplates other nucleotide sequences which encode
the amino acids of SEQ ID NO:2 and SEQ ID NO: 5. It
is within the ken of the ordinarily skilled artisan to
30 identify such sequences which result, for example,
from the degeneracy of the genetic code. Furthermore,

1 one of ordinary skill in the art can determine, by the
gain of function analysis described hereinabove,
smaller subfragments of the fragments containing the
open reading frames which encode $\Delta 6$ -desaturases.

5 The present invention contemplates any such
polypeptide fragment of $\Delta 6$ -desaturase and the nucleic
acids therefor which retain activity for converting LA
to GLA.

In another aspect of the present invention,
10 a vector containing a nucleic acid of the present
invention or a smaller fragment containing the
promoter, coding sequence and termination region of a
 $\Delta 6$ -desaturase gene is transferred into an organism,
for example, cyanobacteria, in which the $\Delta 6$ -desaturase
15 promoter and termination regions are functional.
Accordingly, organisms producing recombinant $\Delta 6$ -
desaturase are provided by this invention. Yet
another aspect of this invention provides isolated $\Delta 6$ -
desaturase, which can be purified from the recombinant
20 organisms by standard methods of protein purification.
(For example, see Ausubel et al. [1987] Current
Protocols in Molecular Biology, Green Publishing
Associates, New York).

Vectors containing DNA encoding $\Delta 6$ -
25 desaturase are also provided by the present invention.
It will be apparent to one of ordinary skill in the
art that appropriate vectors can be constructed to
direct the expression of the $\Delta 6$ -desaturase coding
sequence in a variety of organisms. Replicable
30 expression vectors are particularly preferred.
Replicable expression vectors as described herein are

1 DNA or RNA molecules engineered for controlled
expression of a desired gene, i.e. the $\Delta 6$ -desaturase
gene. Preferably the vectors are plasmids,
bacteriophages, cosmids or viruses. Shuttle vectors,
5 e.g. as described by Wolk et al. (1984) Proc. Natl.
Acad. Sci. USA, 1561-1565 and Bustos et al. (1991) J.
Bacteriol. 174, 7525-7533, are also contemplated in
accordance with the present invention. Sambrook et
al. (1989), Goeddel, ed. (1990) Methods in Enzymology
10 185 Academic Press, and Perbal (1988) A Practical
Guide to Molecular Cloning, John Wiley and Sons, Inc.,
provide detailed reviews of vectors into which a
nucleic acid encoding the present $\Delta 6$ -desaturase can be
inserted and expressed. Such vectors also contain
15 nucleic acid sequences which can effect expression of
nucleic acids encoding $\Delta 6$ -desaturase. Sequence
elements capable of effecting expression of a gene
product include promoters, enhancer elements, upstream
activating sequences, transcription termination
20 signals and polyadenylation sites. Both constitutive
and tissue specific promoters are contemplated. For
transformation of plant cells, the cauliflower mosaic
virus (CaMV) 35S promoter and promoters which are
regulated during plant seed maturation are of
25 particular interest. All such promoter and
transcriptional regulatory elements, singly or in
combination, are contemplated for use in the present
replicable expression vectors and are known to one of
ordinary skill in the art. The CaMV 35S promoter is
30 described, for example, by Restrepo et al. (1990)

1 Plant Cell 2, 987. Genetically engineered and mutated
regulatory sequences are also contemplated.

The ordinarily skilled artisan can determine
vectors and regulatory elements suitable for
5 expression in a particular host cell. For example, a
vector comprising the promoter from the gene encoding
the carboxylase of Anabaena operably linked to the
coding region of $\Delta 6$ -desaturase and further operably
linked to a termination signal from Synechocystis is
10 appropriate for expression of $\Delta 6$ -desaturase in
cyanobacteria. "Operably linked" in this context
means that the promoter and terminator sequences
effectively function to regulate transcription. As a
further example, a vector appropriate for expression
15 of $\Delta 6$ -desaturase in transgenic plants can comprise a
seed-specific promoter sequence derived from
helianthinin, napin, or glycinin operably linked to
the $\Delta 6$ -desaturase coding region and further operably
linked to a seed termination signal or the nopaline
20 synthase termination signal. As a still further
example, a vector for use in expression of $\Delta 6$ -
desaturase in plants can comprise a constitutive
promoter or a tissue specific promoter operably linked
to the $\Delta 6$ -desaturase coding region and further
25 operably linked to a constitutive or tissue specific
terminator or the nopaline synthase termination
signal.

In particular, the helianthinin regulatory
elements disclosed in applicant's copending U.S.
30 Application Serial No. 682,354, filed April 8, 1991
and incorporated herein by reference, are contemplated

- 1 as promoter elements to direct the expression of the
Δ6-desaturase of the present invention.

Modifications of the nucleotide sequences or
regulatory elements disclosed herein which maintain
5 the functions contemplated herein are within the scope
of this invention. Such modifications include
insertions, substitutions and deletions, and
specifically substitutions which reflect the
degeneracy of the genetic code.

- 10 Standard techniques for the construction of
such hybrid vectors are well-known to those of
ordinary skill in the art and can be found in
references such as Sambrook et al. (1989), or any of
the myriad of laboratory manuals on recombinant DNA
15 technology that are widely available. A variety of
strategies are available for ligating fragments of
DNA, the choice of which depends on the nature of the
termini of the DNA fragments. It is further
contemplated in accordance with the present invention
20 to include in the hybrid vectors other nucleotide
sequence elements which facilitate cloning, expression
or processing, for example sequences encoding signal
peptides, a sequence encoding KDEL, which is required
for retention of proteins in the endoplasmic reticulum
25 or sequences encoding transit peptides which direct
Δ6-desaturase to the chloroplast. Such sequences are
known to one of ordinary skill in the art. An
optimized transit peptide is described, for example,
by Van den Broeck et al. (1985) Nature 313, 358.
30 Prokaryotic and eukaryotic signal sequences are

1 disclosed, for example, by Michaelis et al. (1982)
Ann. Rev. Microbiol. 36, 425.

A further aspect of the instant invention provides organisms other than cyanobacteria or plants
5 which contain the DNA encoding the $\Delta 6$ -desaturase of the present invention. The transgenic organisms contemplated in accordance with the present invention include bacteria, cyanobacteria, fungi, and plants and animals. The isolated DNA of the present invention
10 can be introduced into the host by methods known in the art, for example infection, transfection, transformation or transconjugation. Techniques for transferring the DNA of the present invention into such organisms are widely known and provided in
15 references such as Sambrook et al. (1989).

A variety of plant transformation methods are known. The $\Delta 6$ -desaturase gene can be introduced into plants by a leaf disk transformation-regeneration procedure as described by Horsch et al. (1985) Science
20 227, 1229. Other methods of transformation, such as protoplast culture (Horsch et al. (1984) Science 223, 496; DeBlock et al. (1984) EMBO J. 2, 2143; Barton et al. (1983) Cell 32, 1033) can also be used and are within the scope of this invention. In a preferred
25 embodiment plants are transformed with Agrobacterium-derived vectors. However, other methods are available to insert the $\Delta 6$ -desaturase genes of the present invention into plant cells. Such alternative methods include biolistic approaches (Klein et al. (1987) Nature 327, 70), electroporation, chemically-induced
30 DNA uptake, and use of viruses or pollen as vectors.

1 When necessary for the transformation
method, the $\Delta 6$ -desaturase genes of the present
invention can be inserted into a plant transformation
vector, e.g. the binary vector described by Bevan
5 (1984) Nucleic Acids Res. 12, 8111. Plant
transformation vectors can be derived by modifying the
natural gene transfer system of Agrobacterium
tumefaciens. The natural system comprises large Ti
(tumor-inducing)-plasmids containing a large segment,
10 known as T-DNA, which is transferred to transformed
plants. Another segment of the Ti plasmid, the vir
region, is responsible for T-DNA transfer. The T-DNA
region is bordered by terminal repeats. In the
modified binary vectors the tumor-inducing genes have
15 been deleted and the functions of the vir region are
utilized to transfer foreign DNA bordered by the T-DNA
border sequences. The T-region also contains a
selectable marker for antibiotic resistance, and a
multiple cloning site for inserting sequences for
20 transfer. Such engineered strains are known as
"disarmed" A. tumefaciens strains, and allow the
efficient transformation of sequences bordered by the
T-region into the nuclear genomes of plants.

Surface-sterilized leaf disks are inoculated
25 with the "disarmed" foreign DNA-containing A.
tumefaciens, cultured for two days, and then
transferred to antibiotic-containing medium.
Transformed shoots are selected after rooting in
medium containing the appropriate antibiotic,
30 transferred to soil and regenerated.

1 Another aspect of the present invention
provides transgenic plants or progeny of these plants
containing the isolated DNA of the invention. Both
monocotyledenous and dicotyledenous plants are
5 contemplated. Plant cells are transformed with the
isolated DNA encoding $\Delta 6$ -desaturase by any of the
plant transformation methods described above. The
transformed plant cell, usually in a callus culture or
leaf disk, is regenerated into a complete transgenic
10 plant by methods well-known to one of ordinary skill
in the art (e.g. Horsch et al. (1985) Science 227,
1129). In a preferred embodiment, the transgenic
plant is sunflower, oil seed rape, maize, tobacco,
peanut or soybean. Since progeny of transformed
15 plants inherit the DNA encoding $\Delta 6$ -desaturase, seeds
or cuttings from transformed plants are used to
maintain the transgenic plant line.

The present invention further provides a
method for providing transgenic plants with an
20 increased content of GLA. This method includes
introducing DNA encoding $\Delta 6$ -desaturase into plant
cells which lack or have low levels of GLA but contain
LA, and regenerating plants with increased GLA content
from the transgenic cells. In particular,
25 commercially grown crop plants are contemplated as the
transgenic organism, including, but not limited to,
sunflower, soybean, oil seed rape, maize, peanut and
tobacco.

The present invention further provides a
30 method for providing transgenic organisms which
contain GLA. This method comprises introducing DNA

1 encoding $\Delta 6$ -desaturase into an organism which lacks or
has low levels of GLA, but contains LA. In another
embodiment, the method comprises introducing one or
more expression vectors which comprise DNA encoding
5 $\Delta 12$ -desaturase and $\Delta 6$ -desaturase into organisms which
are deficient in both GLA and LA. Accordingly,
organisms deficient in both LA and GLA are induced to
produce LA by the expression of $\Delta 12$ -desaturase, and
GLA is then generated due to the expression of $\Delta 6$ -
10 desaturase. Expression vectors comprising DNA
encoding $\Delta 12$ -desaturase, or $\Delta 12$ -desaturase and $\Delta 6$ -
desaturase, can be constructed by methods of
recombinant technology known to one of ordinary skill
in the art (Sambrook et al., 1989) and the published
15 sequence of $\Delta 12$ -desaturase (Wada et al [1990] Nature
(London) 347, 200-203. In addition, it has been
discovered in accordance with the present invention
that nucleotides 2002-3081 of SEQ. ID NO:1 encode
cyanobacterial $\Delta 12$ -desaturase. Accordingly, this
20 sequence can be used to construct the subject
expression vectors. In particular, commercially grown
crop plants are contemplated as the transgenic
organism, including, but not limited to, sunflower,
soybean, oil seed rape, maize, peanut and tobacco.
25 The present invention is further directed to
a method of inducing chilling tolerance in plants.
Chilling sensitivity may be due to phase transition of
lipids in cell membranes. Phase transition
temperature depends upon the degree of unsaturation of
30 fatty acids in membrane lipids, and thus increasing
the degree of unsaturation, for example by introducing

1 Δ 6-desaturase to convert LA to GLA, can induce or
improve chilling resistance. Accordingly, the present
method comprises introducing DNA encoding Δ 6-
desaturase into a plant cell, and regenerating a plant
5 with improved chilling resistance from said
transformed plant cell. In a preferred embodiment,
the plant is a sunflower, soybean, oil seed rape,
maize, peanut or tobacco plant.

The following examples further illustrate
10 the present invention.

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EXAMPLE 1

Strains and Culture Conditions

Synechocystis (PCC 6803, ATCC 27184),
5 Anabaena (PCC 7120, ATCC 27893) and Synechococcus (PCC
7942, ATCC 33912) were grown photoautotrophically at
30°C in BG11N+ medium (Rippka et al. [1979] J. Gen.
Microbiol. 111, 1-61) under illumination of
incandescent lamps
10 (60 $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{S}^{-1}$). Cosmids and plasmids were selected and
propagated in Escherichia coli strain DH5 α on LB
medium supplemented with antibiotics at standard
concentrations as described by Maniatis et al. (1982)
Molecular Cloning: A Laboratory Manual, Cold Spring
15 Harbor Laboratory, Cold Spring, New York.

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EXAMPLE 2

Construction of Synechocystis Cosmid Genomic Library

5 Total genomic DNA from Synechocystis (PCC 6803) was partially digested with Sau3A and fractionated on a sucrose gradient (Ausubel et al. [1987] Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, New York). Fractions containing 30 to 40 kb DNA fragments
10 were selected and ligated into the dephosphorylated BamHI site of the cosmid vector, pDUCA7 (Buikema et al. [1991] J. Bacteriol. 173, 1879-1885). The ligated DNA was packaged in vitro as described by Ausubel et al. (1987), and packaged phage were propagated in E.
15 coli DH5 α containing the AvaI and Eco4711 methylase helper plasmid, pRL528 as described by Buikema et al. (1991). A total of 1152 colonies were isolated randomly and maintained individually in twelve 96-well microtiter plates.

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EXAMPLE 3

Gain-of-Function Expression of GLA in Anabaena

Anabaena (PCC 7120), a filamentous
5 cyanobacterium, is deficient in GLA but contains
significant amounts of linoleic acid, the precursor
for GLA (Figure 2; Table 2). The Synechocystis cosmid
library described in Example 2 was conjugated into
10 Anabaena (PCC 7120) to identify transconjugants that
produce GLA. Anabaena cells were grown to mid-log
phase in BG11N+ liquid medium and resuspended in the
same medium to a final concentration of approximately
2x10⁸ cells per ml. A mid-log phase culture of E.
15 coli RP4 (Burkardt et al. [1979] J. Gen. Microbiol.
114, 341-348) grown in LB containing ampicillin was
washed and resuspended in fresh LB medium. Anabaena
and RP4 were then mixed and spread evenly on BG11N+
plates containing 5% LB. The cosmid genomic library
was replica plated onto LB plates containing 50 µg/ml
20 kanamycin and 17.5 µg/ml chloramphenicol and was
subsequently patched onto BG11N+ plates containing
Anabaena and RP4. After 24 hours of incubation at
30°C, 30 µg/ml of neomycin was underlaid; and
incubation at 30°C was continued until transconjugants
25 appeared.

Individual transconjugants were isolated
after conjugation and grown in 2 ml BG11N+ liquid
medium with 15 µg/ml neomycin. Fatty acid methyl
esters were prepared from wild type cultures and
30 cultures containing pools of ten transconjugants as
follows. Wild type and transgenic cyanobacterial

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1 cultures were harvested by centrifugation and washed
twice with distilled water. Fatty acid methyl esters
were extracted from these cultures as described by
Dahmer et al. (1989) J. Amer. Oil. Chem. Soc. 66, 543-
5 548 and were analyzed by Gas Liquid Chromatography
(GLC) using a Tracor-560 equipped with a hydrogen
flame ionization detector and capillary column (30 m x
0.25 mm bonded FSOT Superox II, Alltech Associates
Inc., IL). Retention times and co-chromatography of
10 standards (obtained from Sigma Chemical Co.) were used
for identification of fatty acids. The average fatty
acid composition was determined as the ratio of peak
area of each C18 fatty acid normalized to an internal
standard.

15 Representative GLC profiles are shown in
Fig. 2. C18 fatty acid methyl esters are shown.
Peaks were identified by comparing the elution times
with known standards of fatty acid methyl esters and
were confirmed by gas chromatography-mass
20 spectrometry. Panel A depicts GLC analysis of fatty
acids of wild type Anabaena. The arrow indicates the
migration time of GLA. Panel B is a GLC profile of
fatty acids of transconjugants of Anabaena with
pAM542+1.8F. Two GLA producing pools (of 25 pools
25 representing 250 transconjugants) were identified that
produced GLA. Individual transconjugants of each GLA
positive pool were analyzed for GLA production; two
independent transconjugants, AS13 and AS75, one from
each pool, were identified which expressed significant
30 levels of GLA and which contained cosmids, cSy13 and
cSy75, respectively (Figure 3). The cosmids overlap

1 in a region approximately 7.5 kb in length. A 3.5 kb
NheI fragment of cSy75 was recloned in the vector
pDUCA7 and transferred to Anabaena resulting in gain-
of-function expression of GLA (Table 2).

5 Two NheI/Hind III subfragments (1.8 and 1.7
kb) of the 3.5 kb Nhe I fragment of cSy75-3.5 were
subcloned into "pBLUESCRIPT" (Stratagene) (Figure 3)
for sequencing. Standard molecular biology techniques
were performed as described by Maniatis et al. (1982)
10 and Ausubel et al. (1987). Dideoxy sequencing (Sanger
et al. [1977] Proc. Natl. Acad. Sci. USA 74, 5463-
5467) of pBS1.8 was performed with "SEQUENASE" (United
States Biochemical) on both strands by using specific
oligonucleotide primers synthesized by the Advanced
15 DNA Technologies Laboratory (Biology Department, Texas
A & M University). DNA sequence analysis was done
with the GCG (Madison, WI) software as described by
Devereux et al. (1984) Nucleic Acids Res. 12, 387-395.

Both NheI/HindIII subfragments were
20 transferred into a conjugal expression vector, AM542,
in both forward and reverse orientations with respect
to a cyanobacterial carboxylase promoter and were
introduced into Anabaena by conjugation.
Transconjugants containing the 1.8 kb fragment in the
25 forward orientation (AM542-1.8F) produced significant
quantities of GLA and octadecatetraenoic acid (Figure
2; Table 2). Transconjugants containing other
constructs, either reverse oriented 1.8 kb fragment or
forward and reverse oriented 1.7 kb fragment, did not
30 produce detectable levels of GLA (Table 2).

1 Figure 2 compares the C18 fatty acid profile
of an extract from wild type Anabaena (Figure 2A) with
that of transgenic Anabaena containing the 1.8 kb
fragment of cSy75-3.5 in the forward orientation
5 (Figure 2B). GLC analysis of fatty acid methyl esters
from AM542-1.8F revealed a peak with a retention time
identical to that of authentic GLA standard. Analysis
of this peak by gas chromatography-mass spectrometry
(GC-MS) confirmed that it had the same mass
10 fragmentation pattern as a GLA reference sample.
Transgenic Anabaena with altered levels of
polyunsaturated fatty acids were similar to wild type
in growth rate and morphology.

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1 Table 2 Composition of C18 Fatty Acids in Wild Type
and Transgenic Cyanobacteria

5	Strain	Fatty Acid (%)					
		18:0	18:1	18:2	18:3(α)	18:3(γ)	18:4
10	Wild Type						
	<i>Synechocystis</i>	13.6	4.5	54.5	-	27.3	-
	(sp. PCC6803)						
15	<i>Anabaena</i>	2.9	24.8	37.1	35.2	-	-
	(sp. PCC7120)						
	<i>Synechococcus</i>	20.6	79.4	-	-	-	-
	(sp. PCC7942)						
<i>Anabaena</i> Transconjugants							
20	cSy75	3.8	24.4	22.3	9.1	27.9	12.5
	cSy75-3.5	4.3	27.6	18.1	3.2	40.4	6.4
	pAM542 - 1.8F	4.2	13.9	12.1	19.1	25.4	25.4
	pAM542 - 1.8R	7.7	23.1	38.4	30.8	-	-
	pAM542 - 1.7F	2.8	27.8	36.1	33.3	-	-
	pAM542 - 1.7R	2.8	25.4	42.3	29.6	-	-
<i>Synechococcus</i> Transformants							
25	pAM854	27.8	72.2	-	-	-	-
	pAM854 - Δ^{12}	4.0	43.2	46.0	-	-	-
	pAM854 - Δ^6	18.2	81.8	-	-	-	-
	pAM854 - $\Delta^6\Delta^{12}$	42.7	25.3	19.5	-	16.5	-
30	18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid;						
	18:3(α), linolenic acid; 18:3(γ), γ -linolenic acid; 18:4, octadecatetraenoic acid						

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EXAMPLE 4**Transformation of Synechococcus
with $\Delta 6$ and $\Delta 12$ Desaturase Genes**

5 A third cosmid, cSy7, which contains a $\Delta 12$ -
desaturase gene, was isolated by screening the
Synechocystis genomic library with a oligonucleotide
synthesized from the published Synechocystis $\Delta 12$ -
desaturase gene sequence (Wada et al. [1990] Nature
10 (London) 347, 200-203). A 1.7 kb AvaI fragment from
this cosmid containing the $\Delta 12$ -desaturase gene was
identified and used as a probe to demonstrate that
cSy13 not only contains a $\Delta 6$ -desaturase gene but also
a $\Delta 12$ -desaturase gene (Figure 3). Genomic Southern
15 blot analysis further showed that both the $\Delta 6$ -and $\Delta 12$ -
desaturase genes are unique in the Synechocystis
genome so that both functional genes involved in C18
fatty acid desaturation are linked closely in the
Synechocystis genome.

20 The unicellular cyanobacterium Synechococcus
(PCC 7942) is deficient in both linoleic acid and
GLA(3). The $\Delta 12$ and $\Delta 6$ -desaturase genes were cloned
individually and together into pAM854 (Bustos et al.
[1991] J. Bacteriol. 174, 7525-7533), a shuttle vector
that contains sequences necessary for the integration
25 of foreign DNA into the genome of Synechococcus
(Golden et al. [1987] Methods in Enzymol. 153, 215-
231). Synechococcus was transformed with these gene
constructs and colonies were selected. Fatty acid
methyl esters were extracted from transgenic
30 Synechococcus and analyzed by GLC.

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1 Table 2 shows that the principal fatty acids
of wild type Synechococcus are stearic acid (18:0) and
oleic acid (18:1). Synechococcus transformed with
pAM854- Δ 12 expressed linoleic acid (18:2) in addition
5 to the principal fatty acids. Transformants with
pAM854- Δ 6 and Δ 12 produced both linoleate and GLA
(Table 1). These results indicated that Synechococcus
containing both Δ 12- and Δ 6-desaturase genes has
gained the capability of introducing a second double
10 bond at the Δ 12 position and a third double bond at
the Δ 6 position of C18 fatty acids. However, no
changes in fatty acid composition was observed in the
transformant containing pAM854- Δ 6, indicating that in
the absence of substrate synthesized by the Δ 12
15 desaturase, the Δ 6-desaturase is inactive. This
experiment further confirms that the 1.8 kb
NheI/HindIII fragment (Figure 3) contains both coding
and promoter regions of the Synechocystis Δ 6-
desaturase gene. Transgenic Synechococcus with
20 altered levels of polyunsaturated fatty acids were
similar to wild type in growth rate and morphology.

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EXAMPLE 5

Nucleotide Sequence of $\Delta 6$ -Desaturase

The nucleotide sequence of the 1.8 kb fragment of cSy75-3.5 including the functional $\Delta 6$ -desaturase gene was determined. An open reading frame encoding a polypeptide of 359 amino acids was identified (Figure 4). A Kyte-Doolittle hydropathy analysis (Kyte et al. [1982] J. Mol. Biol. 157, 105-132) identified two regions of hydrophobic amino acids that could represent transmembrane domains (Figure 1A); furthermore, the hydropathic profile of the $\Delta 6$ -desaturase is similar to that of the $\Delta 12$ -desaturase gene (Figure 1B; Wada et al.) and $\Delta 9$ -desaturases (Thiede et al. [1986] J. Biol. Chem. 261, 13230-13235). However, the sequence similarity between the Synechocystis $\Delta 6$ - and $\Delta 12$ -desaturases is less than 40% at the nucleotide level and approximately 18% at the amino acid level.

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EXAMPLE 6

Transfer of Cyanobacterial Δ^6 -Desaturase into Tobacco

5 The cyanobacterial Δ^6 -desaturase gene was mobilized into a plant expression vector and transferred to tobacco using Agrobacterium mediated gene transfer techniques. To ensure that the transferred desaturase is appropriately expressed in leaves and developing seeds and that the desaturase

10 gene product is targeted to the endoplasmic reticulum or the chloroplast, various expression cassettes with Synechocystis Δ -desaturase open reading frame (ORF) were constructed. Components of these cassettes include: (i) a 35S promoter or seed specific promoter

15 derived from the sunflower helianthinin gene to drive Δ^6 -desaturase gene expression in all plant tissues or only in developing seeds respectively, (ii) a putative signal peptide either from carrot extensin gene or sunflower helianthinin gene to target newly

20 synthesized Δ^6 -desaturase into the ER, (iii) an ER lumen retention signal sequence (KDEL) at the COOH-terminal of the Δ^6 -desaturase ORF, and (iv) an optimized transit peptide to target Δ^6 desaturase into the chloroplast. The 35S promoter is a derivative of

25 pRTL2 described by Restrepo et al. (1990). The optimized transit peptide sequence is described by Van de Broeck et al. (1985). The carrot extensin signal peptide is described by Chen et al. (1985) EMBO J. 9, 2145.

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Transgenic tobacco plants were produced containing a chimeric cyanobacterial desaturase gene,

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1 comprised of the Synechocystis Δ^6 desaturase gene fused
to an endoplasmic reticulum retention sequence (KDEL)
and extensin signal peptide driven by the CaMV 35S
promoter. PCR amplifications of transgenic tobacco
5 genomic DNA indicate that the Δ^6 desaturase gene was
incorporated into the tobacco genome. Fatty acid
methyl esters of leaves of these transgenic tobacco
plants were extracted and analyzed by Gas Liquid
Chromatography (GLC). These transgenic tobacco
10 accumulated significant amounts of GLA (Figure 4).
Figure 4 shows fatty acid methyl esters as determined
by GLC. Peaks were identified by comparing the
elution times with known standards of fatty acid
methyl ester. Accordingly, cyanobacterial genes
15 involved in fatty acid metabolism can be used to
generate transgenic plants with altered fatty acid
compositions.

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EXAMPLE 7

Construction of Borage cDNA library

5 Membrane bound polysomes were isolated from
borage seeds 12 days post pollination (12 DPP) using
the protocol established for peas by Larkins and
Davies (1975 Plant Phys. 55:749-756). RNA was
extracted from the polysomes as described by Mechler
(1987 Methods in Enzymology 152:241-248, Academic
10 Press).

Poly-A+ RNA was isolated from the membrane
bound polysomal RNA by use of Oligotex-dT beads
(Qiagen). Corresponding cDNA was made using
Stratagene's ZAP cDNA synthesis kit. The cDNA library
15 was constructed in the lambda ZAP II vector
(Stratagene) using the lambda ZAP II vector kit. The
primary library was packaged in Gigapack II Gold
packaging extract (Stratagene). The library was used
to generate expressed sequence tags (ESTs), and
20 sequences corresponding to the tags were used to scan
the GenBank database.

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EXAMPLE 8

Hybridization Protocol

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5 Hybridization probes for screening the
storage cDNA library were generated by using random
primed DNA synthesis as described by Ausubel et al
(1994 Current Protocols in Molecular Biology, Wiley
Interscience, N.Y.) and corresponded to previously
identified abundantly expressed seed storage protein
10 cDNAs. Unincorporated nucleotides were removed by use
of a G-50 spin column (Boehringer Mannheim). Probe was
denatured for hybridization by boiling in a water bath
for 5 minutes, then quickly cooled on ice. Filters
for hybridization were prehybridized at 60°C for 2-4
15 hours in prehybridization solution (6XSSC [Maniatis et
al 1984 Molecular Cloning A Laboratory Manual, Cold
Spring Harbor Laboratory], 1X Denharts Solution, 0.05%
sodium pyrophosphate, 100 µg/ml denatured salmon sperm
DNA). Denatured probe was added to the hybridization
20 solution (6X SSC, 1X Denharts solution, 0.05% sodium
pyrophosphate, 100 µg/ml denatured salmon sperm DNA)
and incubated at 60°C with agitation overnight.
Filters were washed in 4x, 2x, and 1x SET washes for
15 minutes each at 60°C. A 20X SET stock solution is
25 3M NaCl, 0.4 M Tris base, 20 mM Na₂EDTA-2H₂O. The 4X
SET wash was 4X SET, 12.5 mM PO₄, pH 6.8 and 0.2% SDS.
The 2X SET wash was 2X SET, 12.5 mM PO₄, pH 6.8 and
0.2% SDS. The 1X SET wash was 1X SET, 12.5 mM PO₄, pH
6.8 and 0.2% SDS. Filters were allowed to air dry and
30 were then exposed to X-ray film for 24 hours with
intensifying screens at -80°C.

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EXAMPLE 9

Random sequencing of cDNAs from a borage seed
(12 DPP) membrane-bound polysomal library

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The borage cDNA library was plated at low density (500 pfu on 150 mm petri dishes). Highly prevalent seed storage protein cDNAs were "subtracted" by screening with the previously identified corresponding cDNAs. Non-hybridizing plaques were excised using Stratagene's excision protocol and reagents. Resulting bacterial colonies were used to inoculate liquid cultures and were either sequenced manually or by an ABI automated sequencer. Each cDNA was sequenced once and a sequence tag generated from 200-300 base pairs. All sequencing was performed by cycle sequencing (Epicentre). Over 300 ESTs were generated. Each sequence tag was compared to GenBank database by BLASTX computer program and a number of lipid metabolism genes, including the $\Delta 6$ -desaturase were identified.

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Database searches with a cDNA clone designated mbp-65 using BLASTX with the GenBank database resulted in a significant match to the Synechocystis $\Delta 6$ -desaturase. It was determined however, that this clone was not a full length cDNA. A full length cDNA was isolated using mbp-65 to screen the borage membrane-bound polysomal library. The sequence of the isolated cDNA was determined (Fig. 5A, SEQ ID NO:4) and the protein sequence of the open reading frame (Fig. 5B, SEQ ID NO:5) was compared to other known desaturases using Geneworks

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1 (IntelligGenetics) protein alignment program (Fig. 2).
This alignment indicated that the cDNA was the borage
Δ6-desaturase gene.

Although similar to other known plant
5 desaturases, the borage delta 6-desaturase is distinct
as indicated in the dendrogram shown in Fig. 6.
Furthermore, comparison of the amino acid sequences
characteristic of desaturases, particularly those
proposed to be involved in metal binding (metal box 1
10 and metal box 2), illustrates the differences between
the borage delta 6-desaturase and other plant
desaturases (Table 3).

The borage delta 6-desaturase is
distinguished from the cyanobacterial form not only in
15 over all sequence (Fig. 6) but also in the lipid box,
metal box 1 and metal box 2 amino acid motifs (Table
3). As Table 3 indicates, all three motifs are novel
in sequence. Only the borage delta 6-desaturase metal
box 2 shown some relationship to the Synechocystis
20 delta-6 desaturase metal box 2.

In addition, the borage delta 6-desaturase
is also distinct from another borage desaturase gene,
the delta-12 desaturase. P1-81 is a full length cDNA
that was identified by EST analysis and shows high
25 similarity to the Arabidopsis delta-12 desaturase (Fad
2). A comparison of the lipid box, metal box 1 and
metal box 2 amino acid motifs (Table 3) in borage
delta 6 and delta-12 desaturases indicates that little
homology exists in these regions. The placement of
30 the two sequences in the dendrogram in Fig. 6
indicates how distantly related these two genes are.

Table 3. Comparison of common amino acid motifs in membrane-bound desaturases

Desaturase	Amino Acid Motif			
	Lipid Box	Metal Box 1	Metal Box 2	
Borage Δ^6	WIGHDAGH (SEQ. ID. NO: 6)	HNAHH (SEQ. ID. NO: 12)	FQIEHH (SEQ. ID. NO: 20)	
Synechocystis Δ^6	NVGHDANH (SEQ. ID. NO: 7)	HNVLHH (SEQ. ID. NO: 13)	HQVTHH (SEQ. ID. NO: 21)	
Arab. chloroplast Δ^{15}	VLGHDCGH (SEQ. ID. NO: 8)	HRTHH (SEQ. ID. NO: 14)	HVIHH (SEQ. ID. NO: 22)	
Rice Δ^{15}	VLGHDCGH (SEQ. ID. NO: 8)	HRTHH (SEQ. ID. NO: 14)	HVIHH (SEQ. ID. NO: 22)	
Glycine chloroplast Δ^{15}	VLGHDCGH (SEQ. ID. NO: 8)	HRTHH (SEQ. ID. NO: 14)	HVIHH (SEQ. ID. NO: 22)	
Arab. fad3 (Δ^{15})	VLGHDCGH (SEQ. ID. NO: 8)	HRTHH (SEQ. ID. NO: 14)	HVIHH (SEQ. ID. NO: 22)	
Brassica fad3 (Δ^{15})	VLGHDCGH (SEQ. ID. NO: 8)	HRTHH (SEQ. ID. NO: 14)	HVIHH (SEQ. ID. NO: 22)	
Borage Δ^{12} (PI-81)*	VIAHECGH (SEQ. ID. NO: 9)	HRRHH (SEQ. ID. NO: 15)	HVAHH (SEQ. ID. NO: 23)	
Arab. fad2 (Δ^{17})	VIAHECGH (SEQ. ID. NO: 9)	HRRHH (SEQ. ID. NO: 15)	HVAHH (SEQ. ID. NO: 23)	
Arab. chloroplast Δ^{12}	VIGHDCAH (SEQ. ID. NO: 10)	HDRHH (SEQ. ID. NO: 16)	HIPHH (SEQ. ID. NO: 24)	
Glycine plastid Δ^{12}	VIGHDCAH (SEQ. ID. NO: 10)	HDRHH (SEQ. ID. NO: 16)	HIPHH (SEQ. ID. NO: 24)	
Spinach plastidial n-6	VIGHDCAH (SEQ. ID. NO: 10)	HDRHH (SEQ. ID. NO: 16)	HIPHH (SEQ. ID. NO: 24)	
Synechocystis Δ^{12}	VVGHDGCH (SEQ. ID. NO: 11)	HDHHH (SEQ. ID. NO: 18)	HIPHH (SEQ. ID. NO: 24)	
Anabaena Δ^{12}	VLGHDCGH (SEQ. ID. NO: 8)	HNHHH (SEQ. ID. NO: 19)	HVPHH (SEQ. ID. NO: 25)	

*PI-81 is a full length cDNA which was identified by EST analysis and shows high similarity to the Arabidopsis Δ^{12} desaturase (fad2)

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EXAMPLE 10

Construction of 222.1Δ⁶NOS for transient
and expression

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5 The vector pBI221 (Jefferson et al. 1987
EMBO J. 6:3901-3907) was prepared for ligation by
digestion with BamHI and EcoICR I (Promega) which
excises the GUS coding region leaving the 35S promoter
and NOS terminator intact. The borage Δ 6-desaturase
10 cDNA was excised from the Bluescript plasmid
(Stratagene) by digestion with BamHI and XhoI. The
XhoI end was made blunt by use of the Klenow fragment.
This fragment was then cloned into the BamHI/EcoICR I
sites of pBI221, yielding 221.Δ⁶NOS (Fig. 7). In
15 221.Δ⁶.NOS, the remaining portion (backbone) of the
restriction map depicted in Fig. 7 is pBI221.

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EXAMPLE 11

Construction of 121. Δ^6 .NOS for stable transformation

1 The vector pBI121 (Jefferson et al. 1987
5 EMBO J. 6:3901-3907) was prepared for ligation by
digestion with BamHI and EcoICR I (Promega) which
excises the GUS coding region leaving the 35S promoter
and NOS terminator intact. The borage Δ^6 -desaturase
10 cDNA was excised from the Bluescript plasmid
(Stratagene) by digestion with BamHI and XhoI. The
XhoI end was made blunt by use of the Klenow fragment.
This fragment was then cloned into the BamHI/EcoICR I
sites of pBI121, yielding 121.1 Δ^6 NOS (Fig. 7). In
15 121. Δ^6 .NOS, the remaining portion (backbone) of the
restriction map depicted in Fig. 7 is pBI121.

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EXAMPLE 12
Transient Expression

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5 All work involving protoplasts was performed
in a sterile hood. One ml of packed carrot suspension
cells were digested in 30 mls plasmolyzing solution
(25 g/l KCl, 3.5 g/l $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, 10mM MES, pH 5.6 and
0.2 M mannitol) with 1% cellulase, 0.1% pectolyase,
and 0.1% dreisalase overnight, in the dark, at room
10 temperature. Released protoplasts were filtered
through a 150 μm mesh and pelleted by centrifugation
(100x g, 5 min.) then washed twice in plasmolyzing
solution. Protoplasts were counted using a double
chambered hemocytometer. DNA was transfected into the
15 protoplasts by PEG treatment as described by Nunberg
and Thomas (1993 Methods in Plant Molecular Biology
and Biotechnology, B.R. Glick and J.E. Thompson, eds.
pp. 241-248) using 10^6 protoplasts and 50-70 ug of
plasmid DNA (221. Δ 6.NOS). Protoplasts were cultured
20 in 5 mls of MS media supplemented with 0.2M mannitol
and 3 μM 2,4-D for 48 hours in the dark with shaking.

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EXAMPLE 13

Stable transformation of tobacco

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121.Δ⁶.NOS plasmid construction was used to transform tobacco (*Nicotiana tabacum* cv. xanthi) via Agrobacterium according to standard procedures (Horsh et al., 1985 Science 227: 1229-1231; Bogue et al., 1990 Mol. Gen. Genet. 221:49-57), except that initial transformants were selected on 100 ug/ml kanamycin.

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EXAMPLE 14

Preparation and analysis of
fatty acid methyl esters (FAMES)

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5 Tissue from transfected protoplasts and
transformed tobacco plants was frozen in liquid
nitrogen and lyophilized overnight. FAMES were
prepared as described by Dahmer et al (1989 J. Amer.
Oil Chem. Soc. 66:543-548). In some cases, the
solvent was evaporated again, and the FAMES were
10 resuspended in ethyl acetate and extracted once with
deionized water to remove any water soluble
contaminants. The FAMES were analyzed by gas
chromatography (GC) on a J&W Scientific DB-wax column
(30 m length, 0.25 mm ID, 0.25 μ m film).

15 An example of a transient assay is shown in
Fig. 8 which represents three independent
transfections pooled together. The addition of the
borage $\Delta 6$ -desaturase cDNA corresponds with the
appearance of gamma linolenic acid (GLA) which is one
20 of the possible products of $\Delta 6$ -desaturase.

Figures 9 and 10 depict GC profiles of the
FAMES derived from leaf and seed tissue, respectively,
of control and transformed tobacco plants. Figure 9A
provides the profile of leaf tissue of wild-type
25 tobacco (xanthi); Figure 9B provides the profile of
leaf tissue from a tobacco plant transformed with the
borage Δ -6 desaturase under the transcriptional
control of the 35S CaMV promoter (pBI 121 Δ 'NOS).
Peaks correspond to 18:2, 18:3 γ (GLA), 18:3 α and 18:4
30 (octadecanonic acid). Figure 10A shows the GC profile
of seeds of a wild-type tobacco; Figure 10B shows the

- 1 profile of seed tissue of a tobacco plant transformed
with pBI 121 Δ^6 NOS. Peaks correspond to 18:2,
18:3 γ (GLA) and 18:3 α .

- 5 The relative distribution of the C₁₈ fatty
acids in control and transgenic tobacco seeds is shown
in Table 4.

TABLE 4

	Fatty Acid	Xanthi	pBI121 Δ^6 NOS
10	18:0	4.0%	2.5%
	18:1	13%	13%
	18:2	82%	82%
	18:3 γ (GLA)	-	2.7%
15	18:3 α	0.82%	1.4%

- The foregoing results demonstrate that GLA
is incorporated into the triacylglycerides of
transgenic tobacco leaves and seeds containing the
20 borage Δ^6 -desaturase.

25

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Rhone-Poulenc Agrochimie
- (ii) TITLE OF INVENTION: PRODUCTION OF GAMMA LINOLENIC ACID BY A
DELTA 6-DESATURASE
- (iii) NUMBER OF SEQUENCES: 25
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Scully, Scott, Murphy & Presser
 - (B) STREET: 400 Garden City Plaza
 - (C) CITY: Garden City
 - (D) STATE: New York
 - (E) COUNTRY: United States
 - (F) ZIP: 11530
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE: 30-DEC-1994
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Presser, Leopold
 - (B) REGISTRATION NUMBER: 19,827
 - (C) REFERENCE/DOCKET NUMBER: 8383ZYXW
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (516) 742-4343
 - (B) TELEFAX: (516) 742-4366
 - (C) TELEX: 230 901 SANS UR

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 3588 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)

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(ix) FEATURE:

(A) NAME/KEY: CDS
(B) LOCATION: 2002..3081

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GCTAGCCACC AGTGACGATG CCTTGAATTT GGCCATTCTG ACCCAGGCC	GTATTCTGAA	60
TCCCCGCATT CGCATTGTTA ATCGTTTGTT CAACCATGCC CTGGGTAAAC	GTTTAGACAC	120
CACCTTGCCA GACCACGTTA GTTTGAGTGT TTCCGCCCTG GCGGCCCGA	TTTTTTCCTT	180
TGCGGCTTTG GGCAATCAGG CGATCGGGCA ATTGCGTTTG TTTGACCAGA	CTTGGCCCAT	240
TCAGGAAATT GTCATTACCC AAGACCATCC CTGGCTCAAT TTACCCCTGG	CGGATTTATG	300
GGATGATCCG AGCCGAATGT TGATCTATTA CCTACCGGCC CACAGTGAAA	CGGATTTAGT	360
AGGCGCAGTG GTGAATAATT TAACGTTGCA ATCTGGGGAC CATTTAATAG	TGGGACAAAA	420
ACCCCAACCC AAGACCAAAC GCGATCGCC TTGGCGCAA TTTCCAAAC	TGATTACCAA	480
CCTGCGGGAG TATCAGCGGT ATGTCCAACA GGTGATATGG GTGGTGTGT	TTTTATTGTT	540
GATGATTTTT CTGGCCACCT TCATCTACGT TTCCATTGAT CAACATATTG	CCCCAGTGGA	600
CGCGTTGTAT TTTCCGTGG GCATGATTAC CGGGGCCGGT GGCAAGGAAG	AGGTGGCCGA	660
AAAGTCCCCC GATATCATCA AAGTATTCAC AGTGGTGATG ATGATCGCCG	GGGCGGGGGT	720
GATTGGTATT TGTTATGCC TACTGAATGA TTTCATCCTT GGCAGTCGCT	TTAGTCAGTT	780
TTTGGATGCG GCCAAGTTAC CCGATCGCCA TCACATCATC ATTTGTGGGC	TGGGGGGAGT	840
GAGCATGGCC ATTATTGAAG AGTTAATTCA CCAGGGCCAT GAAATTGTGG	TAATCGAAAA	900
GGATACAGAT AATCGTTTCT TGCATACGGC CCGCTCCCTG GGGGTGCCCC	TAATTGTGGA	960
GGATGCCCCG CTAGAAAGAA CGTTGGCCTG CGCCAATATC AACCGAGCCG	AAGCCATTGT	1020
GGTGGCCACC AGCGACGACA CCGTTAACTT GGAAATTGGC CTAATGCCA	AGGCGATCGC	1080
CCCTAGCCTG CCAGTGGTGT TGCCTTGCCA GGATGCCAG TTTAGCCTGT	CCCTGCAGGA	1140
AGTATTTGAA TTTGAAACGG TGCTTTGTCC GGCAGGAATTG GCCACCTATT	CCTTTGCGGC	1200
GGCGGCCCTG GGGGGCAAAA TTTTGGGCAA CGGCATGACC GATGATTGTC	TGTGGGTAGC	1260
CCTAGCCACC TTAATCACTC CTAACCATCC CTTTGCCGAC CAATTGGTTA	AAATTGCAGC	1320
CCAAAAGTCT GATTTCGTTT CCCTCTATCT AGAACGGGGT GGCAAAACCA	TCCATAGCTG	1380
GGAATTATTG GGTACCCATC TCGACTCTGG AGACGTGTTG TATTTAACCA	TGCCC GCCAC	1440
TGCCCTAGAG CAACTTTGGC GATCGCCCCG TGCCACTGCT GATCCTCTGG	ACTCTTTTTT	1500

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GGTTTAGCAT	GGGGGGATGG	AACTCTTGAC	TCGGCCCAAT	GGTGATCAAG	AAAGAACGCT	1560										
TTGTCTATGT	TTAGTATTTT	TAAGTTAACC	AACAGCAGAG	GATAACTTCC	AAAAGAAATT	1620										
AAGCTCAAAA	AGTAGCAAAA	TAAGTTTAAT	TCATAACTGA	GTTTTACTGC	TAAACAGCGG	1680										
TGCAAAAAAG	TCAGATAAAA	TAAAAGCTTC	ACTTCGGTTT	TATATTGTGA	CCATGGTTCC	1740										
CAGGCATCTG	CTCTAGGGAG	TTTTTCCGCT	GCCTTTAGAG	AGTATTTTCT	CCAAGTCGGC	1800										
TAACTCCCCC	ATTTTTAGGC	AAAATCATAT	ACAGACTATC	CCAATATTGC	CAGAGCTTTG	1860										
ATGACTCACT	GTAGAAGGCA	GAATAAAATT	CTAGCAATGG	ACTCCCAGTT	GGAATAAATT	1920										
TTTAGTCTCC	CCCGGCGCTG	GAGTTTTTTT	GTAGTTAATG	GCGGTATAAT	GTGAAAGTTT	1980										
TTTATCTATT	TAAATTTATA	A	ATG	CTA	ACA	GCG	GAA	AGA	ATT	AAA	TTT	ACC	2031			
			Met	Leu	Thr	Ala	Glu	Arg	Ile	Lys	Phe	Thr				
			1				5					10				
CAG	AAA	CGG	GGG	TTT	CGT	CGG	GTA	CTA	AAC	CAA	CGG	GTG	GAT	GCC	TAC	2079
Gln	Lys	Arg	Gly	Phe	Arg	Arg	Val	Leu	Asn	Gln	Arg	Val	Asp	Ala	Tyr	
			15						20					25		
TTT	GCC	GAG	CAT	GGC	CTG	ACC	CAA	AGG	GAT	AAT	CCC	TCC	ATG	TAT	CTG	2127
Phe	Ala	Glu	His	Gly	Leu	Thr	Gln	Arg	Asp	Asn	Pro	Ser	Met	Tyr	Leu	
			30					35					40			
AAA	ACC	CTG	ATT	ATT	GTG	CTC	TGG	TTG	TTT	TCC	GCT	TGG	GCC	TTT	GTG	2175
Lys	Thr	Leu	Ile	Ile	Val	Leu	Trp	Leu	Phe	Ser	Ala	Trp	Ala	Phe	Val	
		45					50					55				
CTT	TTT	GCT	CCA	GTT	ATT	TTT	CCG	GTG	CGC	CTA	CTG	GGT	TGT	ATG	GTT	2223
Leu	Phe	Ala	Pro	Val	Ile	Phe	Pro	Val	Arg	Leu	Leu	Gly	Cys	Met	Val	
	60					65					70					
TTG	GCG	ATC	GCC	TTG	GCG	GCC	TTT	TCC	TTC	AAT	GTC	GGC	CAC	GAT	GCC	2271
Leu	Ala	Ile	Ala	Leu	Ala	Ala	Phe	Ser	Phe	Asn	Val	Gly	His	Asp	Ala	
	75				80					85					90	
AAC	CAC	AAT	GCC	TAT	TCC	TCC	AAT	CCC	CAC	ATC	AAC	CGG	GTT	CTG	GGC	2319
Asn	His	Asn	Ala	Tyr	Ser	Ser	Asn	Pro	His	Ile	Asn	Arg	Val	Leu	Gly	
			95					100						105		
ATG	ACC	TAC	GAT	TTT	GTC	GGG	TTA	TCT	AGT	TTT	CTT	TGG	CGC	TAT	CGC	2367
Met	Thr	Tyr	Asp	Phe	Val	Gly	Leu	Ser	Ser	Phe	Leu	Trp	Arg	Tyr	Arg	
			110					115					120			
CAC	AAC	TAT	TTG	CAC	CAC	ACC	TAC	ACC	AAT	ATT	CTT	GGC	CAT	GAC	GTG	2415
His	Asn	Tyr	Leu	His	His	Thr	Tyr	Thr	Asn	Ile	Leu	Gly	His	Asp	Val	
		125					130					135				
GAA	ATC	CAT	GGA	GAT	GGC	GCA	GTA	CGT	ATG	AGT	CCT	GAA	CAA	GAA	CAT	2463
Glu	Ile	His	Gly	Asp	Gly	Ala	Val	Arg	Met	Ser	Pro	Glu	Gln	Glu	His	
	140					145					150					

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TTTGAGGGGG TTCATTGGCC GCAGTTTCAA GCTGACCTAG GAGGCAAAGA TTGGGTGATT 3268
TTGCTCAAAT CCGCTGGGAT ATTGAAAGGC TTCACCACCT TTGGTTTCTA CCCTGCTCAA 3328
TGGGAAGGAC AAACCGTCAG AATTGTTTAT TCTGGTGACA CCATCACCGA CCCATCCATG 3388
TGGTCTAACC CAGCCCTGGC CAAGGCTTGG ACCAAGGCCA TGCAAATTCT CCACGAGGCT 3448
AGGCCAGAAA AATTATATTG GCTCCTGATT TCTTCCGGCT ATCGCACCTA CCGATTTTTG 3508
AGCATTTTTG CCAAGGAATT CTATCCCCAC TATCTCCATC CCACTCCCCC GCCTGTACAA 3568
AATTTTATCC ATCAGCTAGC 3588

```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 359 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```

Met Leu Thr Ala Glu Arg Ile Lys Phe Thr Gln Lys Arg Gly Phe Arg
 1           5           10           15
Arg Val Leu Asn Gln Arg Val Asp Ala Tyr Phe Ala Glu His Gly Leu
          20           25           30
Thr Gln Arg Asp Asn Pro Ser Met Tyr Leu Lys Thr Leu Ile Ile Val
          35           40           45
Leu Trp Leu Phe Ser Ala Trp Ala Phe Val Leu Phe Ala Pro Val Ile
          50           55           60
Phe Pro Val Arg Leu Leu Gly Cys Met Val Leu Ala Ile Ala Leu Ala
          65           70           75           80
Ala Phe Ser Phe Asn Val Gly His Asp Ala Asn His Asn Ala Tyr Ser
          85           90           95
Ser Asn Pro His Ile Asn Arg Val Leu Gly Met Thr Tyr Asp Phe Val
          100          105          110
Gly Leu Ser Ser Phe Leu Trp Arg Tyr Arg His Asn Tyr Leu His His
          115          120          125
Thr Tyr Thr Asn Ile Leu Gly His Asp Val Glu Ile His Gly Asp Gly
          130          135          140
Ala Val Arg Met Ser Pro Glu Gln Glu His Val Gly Ile Tyr Arg Phe
          145          150          155          160

```

(A) LENGTH: 1884 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

AGCTTCACTT CGGTTTTATA TTGTGACCAT GGTTCCCAGG CATCTGCTCT AGGGAGTTTT 60
TCCGCTGCCT TTAGAGAGTA TTTTCTCCAA GTCGGCTAAC TCCCCCATTT TTAGGCCAAA 120

TCATATACAG	ACTATCCCAA	TATTGCCAGA	GCTTTGATGA	CTCACTGTAG	AAGGCAGACT	180
AAAATTCTAG	CAATGGACTC	CCAGTTGGAA	TAAATTTTAA	GTCTCCCCCG	GCGCTGGAGT	240
TTTTTTGTAG	TTAATGGCGG	TATAATGTGA	AAGTTTTTTA	TCTATTTAAA	TTTATAAATG	300
CTAACAGCGG	AAAGAATTAA	ATTTACCCAG	AAACGGGGGT	TTCGTCGGGT	ACTAAACCAA	360
CGGGTGGATG	CCTACTTTGC	CGAGCATGGC	CTGACCCAAA	GGGATAATCC	CTCCATGTAT	420
CTGAAAACCC	TGATTATTGT	GCTCTGGTTG	TTTTCCGCTT	GGGCCTTTGT	GCTTTTTGCT	480
CCAGTTATTT	TTCCGGTGCG	CCTACTGGGT	TGTATGGTTT	TGGCGATCGC	CTTGGCGGCC	540
TTTTCTTCA	ATGTCGGCCA	CGATGCCAAC	CACAATGCCT	ATTCTCCAA	TCCCCACATC	600
AACCGGGTTC	TGGGCATGAC	CTACGATTTT	GTCGGGTTAT	CTAGTTTTCT	TTGGCGCTAT	660
CGCCACAAC	ATTTGCACCA	CACCTACACC	AATATTCTTG	GCCATGACGT	GGAAATCCAT	720
GGAGATGGCG	CAGTACGTAT	GAGTCCTGAA	CAAGAACATG	TTGGTATTTA	TCGTTTCCAG	780
CAATTTTATA	TTTGGGGTTT	ATATCTTTTC	ATTCCCTTTT	ATTGGTTTCT	CTACGATGTC	840
TACCTAGTGC	TTAATAAAGG	CAAATATCAC	GACCATAAAA	TTCTCCTTT	CCAGCCCCTA	900
GAATTAGCTA	GTTTGCTAGG	GATTAAGCTA	TTATGGCTCG	GCTACGTTTT	CGGCTTACCT	960
CTGGCTCTGG	GCTTTTCCAT	TCCTGAAGTA	TTAATTGGTG	CTTCGGTAAC	CTATATGACC	1020
TATGGCATCG	TGGTTTGCAC	CATCTTTATG	CTGGCCCATG	TGTTGGAATC	AACTGAATTT	1080
CTCACCCCG	ATGGTGAATC	CGGTGCCATT	GATGACGAGT	GGGCTATTTG	CCAAATTCGT	1140
ACCACGGCCA	ATTTTGCCAC	CAATAATCCC	TTTTGGAACT	GGTTTTGTGG	CGGTTTAAAT	1200
CACCAAGTTA	CCCACCATCT	TTTCCCAAT	ATTTGTCATA	TTCATATCC	CCAAATTGGAA	1260
AATATTATTA	AGGATGTTTG	CCAAGAGTTT	GGTGTGGAAT	ATAAAGTTTA	TCCCACCTTC	1320
AAAGCGGCGA	TCGCCTCTAA	CTATCGCTGG	CTAGAGGCCA	TGGGCAAAGC	ATCGTGACAT	1380
TGCCTTGGA	TTGAAGCAAA	ATGGCAAAAT	CCCTCGTAAA	TCTATGATCG	AAGCCTTTCT	1440
GTTGCCCGCC	GACCAAATCC	CCGATGCTGA	CCAAAGGTTG	ATGTTGGCAT	TGCTCCAAAC	1500
CCACTTTGAG	GGGGTTCATT	GGCCGCAGTT	TCAAGCTGAC	CTAGGAGGCA	AAGATTGGGT	1560
GATTTTGCTC	AAATCCGCTG	GGATATTGAA	AGGCTTCACC	ACCTTTGGTT	TCTACCTGTC	1620
TCAATGGGAA	GGACAAACCG	TCAGAATTGT	TTATTCTGGT	GACACCATCA	CCGACCCATC	1680
CATGTGGTCT	AACCCAGCCC	TGGCCAAGGC	TTGGACCAAG	GCCATGCAAA	TTCTCCACGA	1740
GGCTAGGCCA	GAAAAATTAT	ATTGGCTCCT	GATTTCTTCC	GGCTATCGCA	CCTACCGATT	1800

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TTTGAGCATT TTGCCAAGG AATTCTATCC CCACTATCTC CATCCCACTC CCCCGCCTGT 1860

ACAAAATTTT ATCCATCAGC TAGC 1884

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1685 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AATATCTGCC TACCCTCCCA AAGAGAGTAG TCATTTTTC A	60
GAAATACATT ACCTCAGATG AACTCAAGAA CCACGATAAA CCCGGAGATC TATGGATCTC	120
GATTCAAGGG AAAGCCTATG ATGTTTCGGA TTGGGTGAAA GACCATCCAG GTGGCAGCTT	180
TCCCTTGAAG AGTCTTGCTG GTCAAGAGGT AACTGATGCA TTTGTTGCAT TCCATCCTGC	240
CTCTACATGG AAGAATCTTG ATAAGTTTTT CACTGGGTAT TATCTTAAAG ATTACTCTGT	300
TTCTGAGGTT TCTAAAGATT ATAGGAAGCT TGTGTTTGAG TTTTCTAAAA TGGGTTTGTA	360
TGACAAAAAA GGTCATATTA TGTTTGCAAC TTTGTGCTTT ATAGCAATGC TGTTTGCTAT	420
GAGTGTTTAT GGGGTTTTGT TTTGTGAGGG TGTTTTGGTA CATTTGTTTT CTGGGTGTTT	480
GATGGGGTTT CTTTGGATTC AGAGTGGTTG GATTGGACAT GATGCTGGGC ATTATATGGT	540
AGTGTCTGAT TCAAGGCTTA ATAAGTTTAT GGGTATTTTT GCTGCAAATT GTCTTTCAGG	600
AATAAGTATT GGTTGGTGGA AATGGAACCA TAATGCACAT CACATTGCCT GTAATAGCCT	660
TGAATATGAC CCGATTTTAC AATATATACC ATTCCTTGTT GTGTCTTCCA AGTTTTTTGG	720
TTCACTCACC TCTCATTTCT ATGAGAAAAG GTTGACTTTT GACTCTTTAT CAAGATTCTT	780
TGTAAGTTAT CAACATTGGA CATTTTACCC TATTATGTGT GCTGCTAGGC TCAATATGTA	840
TGTACAATCT CTCATAATGT TGTTGACCAA GAGAAATGTG TCCTATCGAG CTCAGGAACT	900
CTTGGGATGC CTAGTGTTCT CGATTTGGTA CCCGTTGCTT GTTTCTTGTT TGCCTAATTG	960
GGGTGAAAGA ATTATGTTTG TTATTGCAAG TTTATCAGTG ACTGGAATGC AACAAGTTCA	1020
GTTCTCCTTG AACCACTTCT CTTCAAGTGT TTATGTTGGA AAGCCTAAAG GGAATAATTG	1080
GTTTGAGAAA CAAACGGATG GGACACTTGA CATTTCTTGT CTCCTTGGA TGGATTGGTT	1140
TCATGGTGGA TTGCAATTCC AAATTGAGCA TCATTGTTT CCCAAGATGC CTAGATGCAA	1200

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CCTTAGGAAA ATCTCGCCCT ACGTGATCGA GTTATGCAAG AACATAATT TGCCTTACAA      1260
TTATGCATCT TTCTCCAAGG CCAATGAAAT GACACTCAGA ACATTGAGGA ACACAGCATT      1320
GCAGGCTAGG GATATAACCA AGCCGCTCCC GAAGAATTTG GTATGGGAAG CTCTTCACAC      1380
TCATGGTTAA AATTACCCTT AGTTCATGTA ATAATTTGAG ATTATGTATC TCCTATGTTT      1440
GTGTCCTTGTC TTGGTTCTAC TTGTTGGAGT CATTGCAACT TGTCTTTTAT GGTTTATTAG      1500
ATGTTTTTTTA ATATATTTTA GAGGTTTTGC TTTCATCTCC ATTATTGATG AATAAGGAGT      1560
TGCATATTGT CAATTGTTGT GCTCAATATC TGATATTTTG GAATGTACTT TGTACCACTG      1620
TGTTTTTCAGT TGAAGCTCAT GTGTACTTCT ATAGACTTTG TTTAAATGGT TATGTCATGT      1680
TATTT                                         1685

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(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 448 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```

Met Ala Ala Gln Ile Lys Lys Tyr Ile Thr Ser Asp Glu Leu Lys Asn
 1             5             10             15
His Asp Lys Pro Gly Asp Leu Trp Ile Ser Ile Gln Gly Lys Ala Tyr
 20             25             30
Asp Val Ser Asp Trp Val Lys Asp His Pro Gly Gly Ser Phe Pro Leu
 35             40             45
Lys Ser Leu Ala Gly Gln Glu Val Thr Asp Ala Phe Val Ala Phe His
 50             55             60
Pro Ala Ser Thr Trp Lys Asn Leu Asp Lys Phe Phe Thr Gly Tyr Tyr
 65             70             75             80
Leu Lys Asp Tyr Ser Val Ser Glu Val Ser Lys Asp Tyr Arg Lys Leu
 85             90             95
Val Phe Glu Phe Ser Lys Met Gly Leu Tyr Asp Lys Lys Gly His Ile
 100            105            110
Met Phe Ala Thr Leu Cys Phe Ile Ala Met Leu Phe Ala Met Ser Val
 115            120            125
Tyr Gly Val Leu Phe Cys Glu Gly Val Leu Val His Leu Phe Ser Gly
 130            135            140

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Cys Leu Met Gly Phe Leu Trp Ile Gln Ser Gly Trp Ile Gly His Asp
 145 150 155 160
 Ala Gly His Tyr Met Val Val Ser Asp Ser Arg Leu Asn Lys Phe Met
 165 170 175
 Gly Ile Phe Ala Ala Asn Cys Leu Ser Gly Ile Ser Ile Gly Trp Trp
 180 185 190
 Lys Trp Asn His Asn Ala His His Ile Ala Cys Asn Ser Leu Glu Tyr
 195 200 205
 Asp Pro Asp Leu Gln Tyr Ile Pro Phe Leu Val Val Ser Ser Lys Phe
 210 215 220
 Phe Gly Ser Leu Thr Ser His Phe Tyr Glu Lys Arg Leu Thr Phe Asp
 225 230 235 240
 Ser Leu Ser Arg Phe Phe Val Ser Tyr Gln His Trp Thr Phe Tyr Pro
 245 250 255
 Ile Met Cys Ala Ala Arg Leu Asn Met Tyr Val Gln Ser Leu Ile Met
 260 265 270
 Leu Leu Thr Lys Arg Asn Val Ser Tyr Arg Ala Gln Glu Leu Leu Gly
 275 280 285
 Cys Leu Val Phe Ser Ile Trp Tyr Pro Leu Leu Val Ser Cys Leu Pro
 290 295 300
 Asn Trp Gly Glu Arg Ile Met Phe Val Ile Ala Ser Leu Ser Val Thr
 305 310 315 320
 Gly Met Gln Gln Val Gln Phe Ser Leu Asn His Phe Ser Ser Ser Val
 325 330 335
 Tyr Val Gly Lys Pro Lys Gly Asn Asn Trp Phe Glu Lys Gln Thr Asp
 340 345 350
 Gly Thr Leu Asp Ile Ser Cys Pro Pro Trp Met Asp Trp Phe His Gly
 355 360 365
 Gly Ser Gln Phe Gln Ile Glu His His Leu Phe Pro Lys Met Pro Arg
 370 375 380
 Cys Asn Leu Arg Lys Ile Ser Pro Tyr Val Ile Glu Leu Cys Lys Lys
 385 390 395 400
 His Asn Leu Pro Tyr Asn Tyr Ala Ser Phe Ser Lys Ala Asn Glu Met
 405 410 415
 Thr Leu Arg Thr Leu Arg Asn Thr Ala Leu Gln Ala Arg Asp Ile Thr
 420 425 430
 Lys Pro Leu Pro Lys Asn Leu Val Trp Glu Ala Leu His Thr His Gly
 435 440 445

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(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Trp Ile Gly His Asp Ala Gly His
1 5

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Asn Val Gly His Asp Ala Asn His
1 5

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Val Leu Gly His Asp Cys Gly His
1 5

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

-55-

Val Ile Ala His Glu Cys Gly His
1 5

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Val Ile Gly His Asp Cys Ala His
1 5

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Val Val Gly His Asp Cys Gly His
1 5

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

His Asn Ala His His
1 5

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

-56-

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

His Asn Tyr Leu His His
1 5

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

His Arg Thr His His
1 5

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

His Arg Arg His His
1 5

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

His Asp Arg His His
1 5

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

His Asp Gln His His
1 5

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

His Asp His His His
1 5

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

His Asn His His His
1 5

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Phe Gln Ile Glu His His
1 5

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

His Gln Val Thr His His
1 5

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

His Val Ile His His
1 5

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids
(B) TYPE: amino acid

-59-

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

His Val Ala His His
1 5

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

His Ile Pro His His
1 5

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

His Val Pro His His
1 5

1 WHAT IS CLAIMED:

1. An isolated nucleic acid encoding a borage $\Delta 6$ -desaturase.

5

2. The isolated nucleic acid of Claim 1 comprising the nucleotide sequence of SEQ ID NO: 4.

3. An isolated nucleic acid that codes for the amino acid sequence of SEQ ID NO: 5.

10

4. A vector comprising the nucleic acid of any one Claims 1-3.

15

5. An expression vector comprising the isolated nucleic acid of any one of Claims 1-3 operably linked to a promoter and optionally a termination signal capable of effecting expression of the gene product of said isolated nucleic acid.

20

6. The expression vector of Claim 5 wherein said promoter is a Δ -6 desaturase promoter, an Anabaena carboxylase promoter, a helianthinin promoter, a glycinin promoter, a napin promoter, the 35S promoter from CaMV, or a helianthinin tissue-specific promoter.

25

7. The expression vector of Claim 5 wherein said promoter is constitutive or tissue-specific.

30

8. The expression vector of Claim 5 wherein said termination signal is a Synechocystis termination

35

1 signal, a nopaline synthase termination signal, or a seed
termination signal.

5 9. A cell comprising the vector of any one of
Claims 4-8.

10 10. The cell of Claim 9 wherein said cell is an
animal cell, a bacterial cell, a plant cell or a fungal
cell.

11. A transgenic organism comprising the
isolated nucleic acid of any one of Claims 1-3.

15 12. A transgenic organism comprising the vector
of any one of Claims 4-8.

13. The transgenic organism of Claim 11 or 12
wherein said organism is a bacterium, a fungus, a plant or
an animal.

20 14. A plant or progeny of said plant which has
been regenerated from the plant cell of Claim 10.

25 15. The plant of Claim 14 wherein said plant is
a sunflower, soybean, maize, tobacco, peanut, carrot or
oil seed rape plant.

30 16. A method of producing a plant with
increased gamma linolenic acid (GLA) content which
comprises:

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- 1 (a) transforming a plant cell with the isolated
nucleic acid of any one of Claims 1-3; and
(b) regenerating a plant with increased GLA
content from said plant cell.

5

17. A method of producing a plant with
increased gamma linolenic acid (GLA) content which
comprises:

- (a) transforming a plant cell with the vector of
10 any one of Claims 4-8; and
(b) regenerating a plant with increased GLA
content from said plant cell.

18. The method of Claim 16 or 17 wherein said
15 plant is a sunflower, soybean, maize, tobacco, peanut,
carrot or oil seed rape plant.

19. A method of inducing production of gamma
linolenic acid (GLA) in an organism deficient or lacking
20 in GLA which comprises transforming said organism with the
isolated nucleic acid of any one of Claims 1-3.

20. A method of inducing production of gamma
linolenic acid (GLA) in an organism deficient or lacking
25 in GLA which comprises transforming said organism with the
vector of any one of Claims 4-8.

21. A method of inducing production of gamma
linolenic acid (GLA) in an organism deficient or lacking
30 in GLA and linoleic acid (LA) which comprises transforming
said organism with an isolated nucleic acid encoding

35

1 borage $\Delta 6$ -desaturase and an isolated nucleic acid encoding
12-desaturase.

22. The method of Claim 21 wherein said
5 isolated nucleic acid encoding $\Delta 6$ -desaturase comprises
nucleotides 44 to 1390 of SEQ. ID NO: 4.

23. A method of inducing production of
octadecatetraeonic acid in an organism deficient or
10 lacking in gamma linolenic acid which comprises
transforming said organism with the isolated nucleic acid
of any one of Claims 1-3.

24. A method of inducing production of
15 octadecatetraeonic acid in an organism deficient or
lacking in gamma linolenic acid which comprises
transforming said organism with the vector of any one of
Claims 4-8.

20 25. The method of Claim 23 or 24 wherein said
organism is a bacterium, a fungus, a plant or an animal.

26. A method of producing a plant with improved
chilling resistance which comprises:
25 (a) transforming a plant cell with the isolated
nucleic acid of any one of Claims 1-3; and
(b) regenerating said plant with improved
chilling resistance from said transformed plant cell.

30 27. A method of producing a plant with improved
chilling resistance which comprises:

-64-

1 (a) transforming a plant cell with the vector of
any one of Claims 4-8; and

(b) regenerating said plant with improved
chilling resistance from said transformed plant cell.

5

28. The method of Claim 26 or 27 wherein said
plant is a sunflower, soybean, maize, tobacco, peanut,
carrot or oil seed rape plant.

10

15

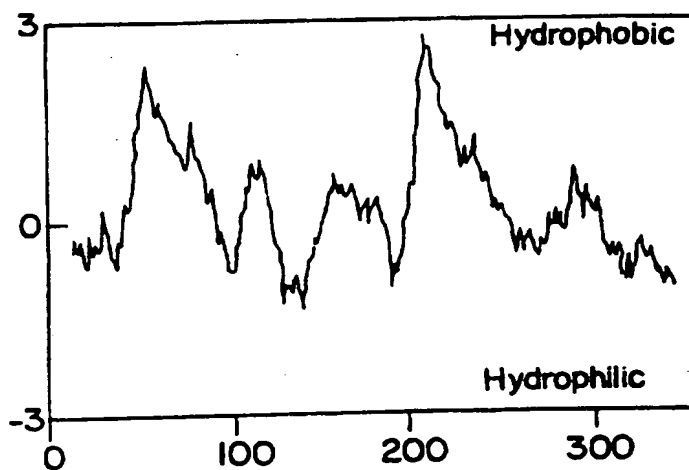
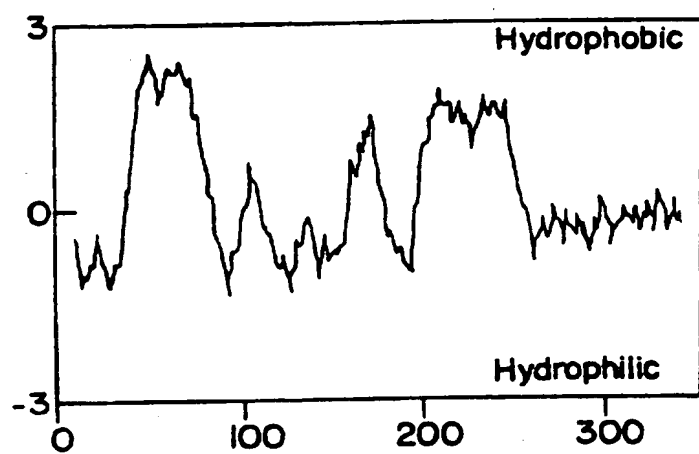
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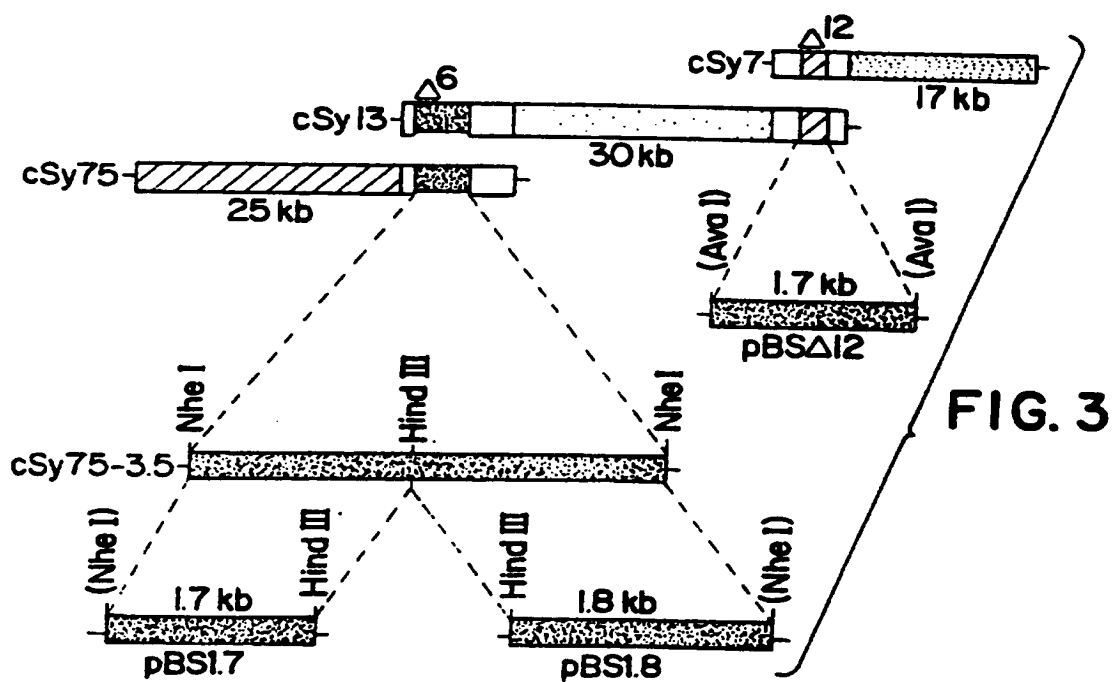
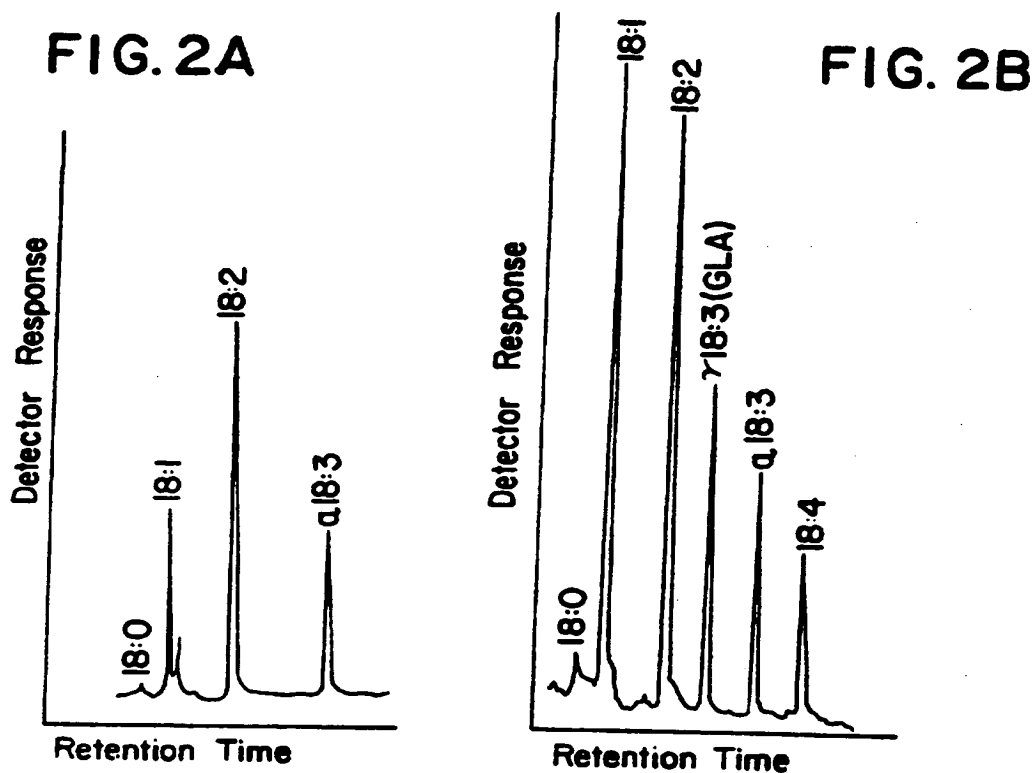
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SUBSTITUTE SHEET (RULE 26)

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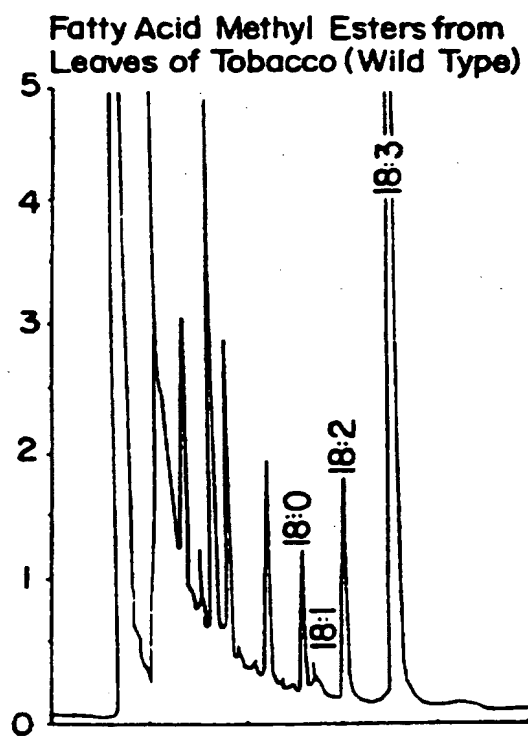


FIG. 4A

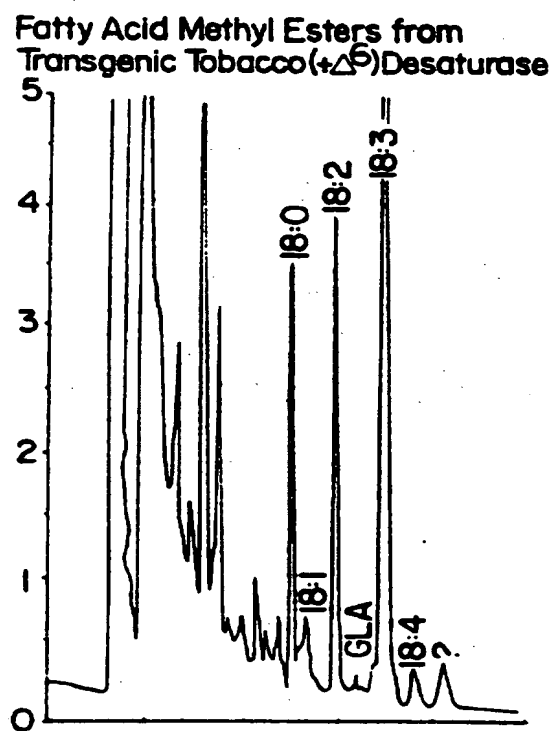


FIG. 4B

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FIG. 5A

1 aatatctgcc taccctccca aagagagtag tcatctttca
 81 aactcaagaa ccacgataaa cccggagatc tatggatctc
 161 gaccatccag gtggcagctt tcccttgaag agtcttgctg
 241 ctctacatgg aagaatcttg ataatgtttt cactgggtat
 321 ataggaagct tgtgtttgag ttttctaaaa tgggtttgta
 401 atagcaatgc tgtttgctat gagtgtttat ggggttttgt
 481 gatgggggtt ctttggattc agagtgggtg gatggacat
 561 ataatgtttat ggggtatttt gctgcaaatt gcttttcagg
 641 cacattgcct gtaatagcct tgaatatgac cctgatttac
 721 ttcactcacc tctcatttct atgagaaaag gttgactttt
 801 ctttttacc tattatgtgt gctgtaggc tcaatatgta
 881 tcttatcgag ctccaggaaact cttgggatgc ctagtgttct
 961 ggggtgaaaga attatgtttg ttattgcaag ttatcagtg
 1041 cttcaagtgt ttatgttggga aagcctaaag ggaataattg
 1121 cctccttggga tggattgggt ttatgttggga ttgcaattcc
 1201 ctttaggaaa atctcgccct acgtgatcga gttatgcaag
 1281 ccaatgaaat gacactcaga acattgagga acacagcatt
 1361 gtatgggaag ctcttcacac tcatgggttaa aattaccctt
 1441 gtgtcttgct ttggttctac ttgttggagt cattgcaact
 1521 gaggttttgc ttctatctcc attattgatg aataaggagt
 1601 gaatgtactt tgtaccactg tgttttcagt tgaagctcat
 1681 tattt
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 aataagtatt ggttgggtga aatggaacca taatgcacat 640
 aatatatacc attccttgtt gtgtcttcca agtttttgg 720
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 tgtacaatct ctcataatgt tgttgaccaa gagaaatgtg 880
 cgatttgga cccgttgctt gtttcttgtt tgcctaattg 960
 actggaatgc aacaagtcca gttctccttg aaccattct 1040
 gtttgagaaa caaacggatg ggacacttga catttcttgt 1120
 aaattgagca tcatgtgtt cccaagatgc ctatagtgcaa 1200
 aaacataatt tgccttacia ttatgcatct ttctccaagg 1280
 gcaggctagg gatataacca agcgcctccc gaagaatttg 1360
 agtctcatgta ataatttgag attatgtatc tcctatgttt 1440
 tgtcttttat ggtttattag atgttttata atatatatta 1520
 tgcataattgt caattgttgt gctcaaatc tgataatttg 1600
 gtgtacttct atagactttg tttaaatggc tatgtcatgt 1685

FIG. 5B

1 MAAQIKKYIT SDELKNHDKP GDLWISIQGK AYDVSDWVKD HPGSFFPLKS LAGQEVTDFA VAFHPASTWK NLDKFFFTGY 80
 81 LKDYSVSEVS KDYRKLVEEF SKMGLYDKKG HIMFATLCFI AMLFAMSVYG VLFCEGVLVH LFSGCLMGFL WIQSGWIGHD 160
 161 AGHYMVVSDS RLNKFMGIFA ANCLSGISIG WKKWNHNAHH IACNSLEYDP DLQYIPFLVV SSKFFGSLTS HFYEKRLTFD 240
 241 SLRFFVSYQ HWTFYPIMCA ARLNMYVQSL IMLLTKRNV YRAQELLGCL VFSIWYPLLV SCLPNWGERI MFVIASLSVT 320
 321 GMOQVQFSLN HFSSSVYVGK PKGNWFEKQ TDGTLDISCP PMDWFHGGL QFQIEHHLFP KNPRCNLRKI SPYVIELCKK 400
 401 HNLPPNYASF SKANEMTLRT LRNTALQARD ITKPLPKNLV WEALHTHG 448

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FIG. 6

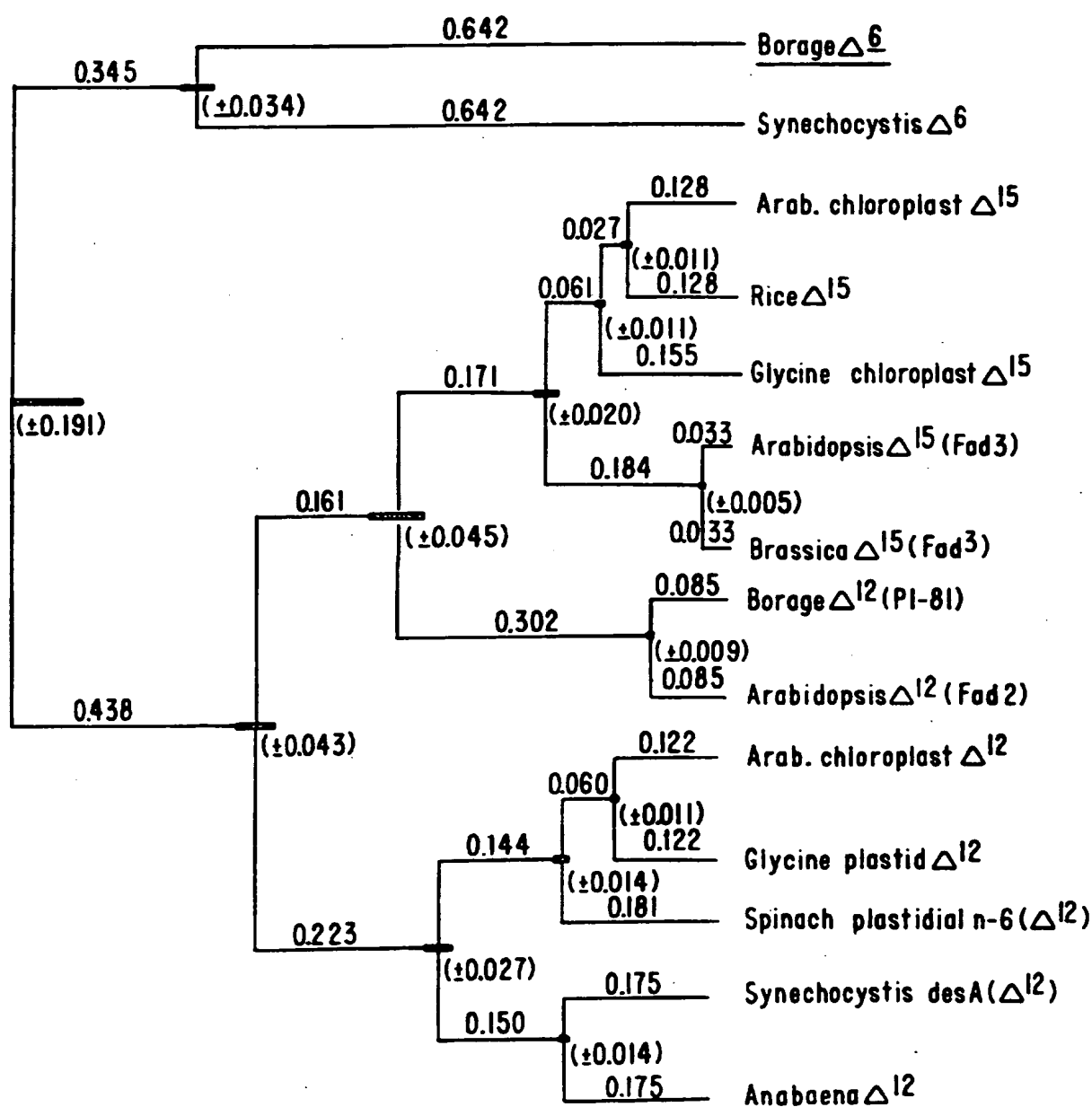


FIG. 7

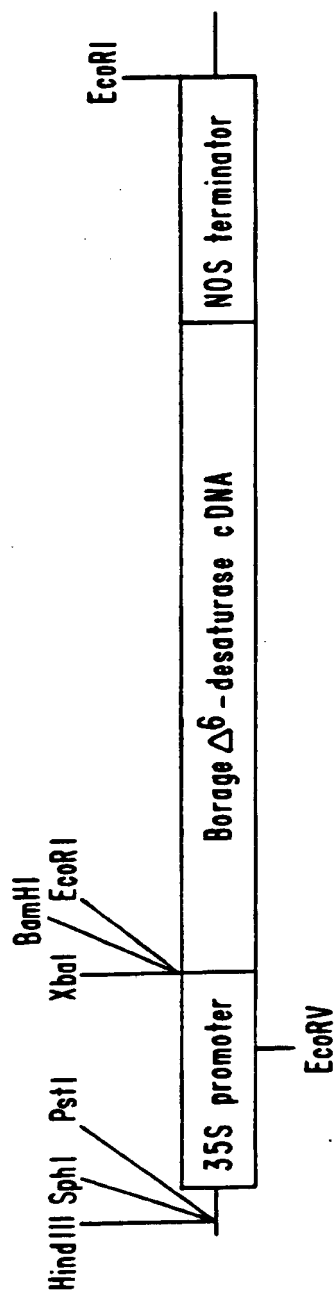


FIG. 8A

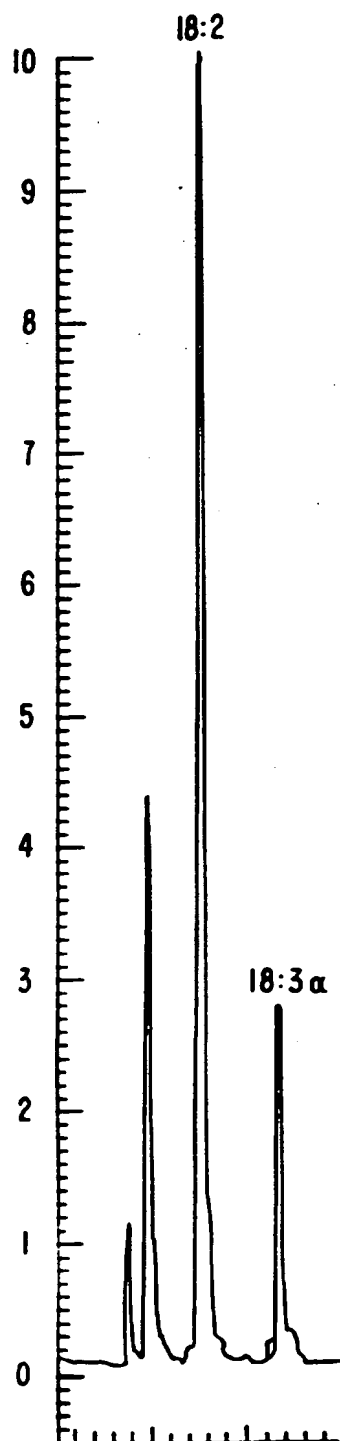


FIG. 8B

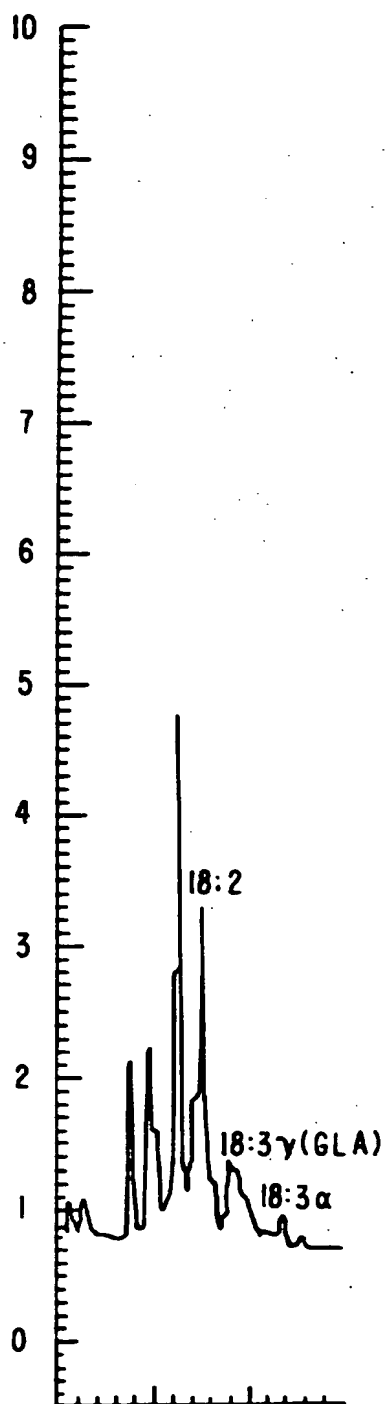
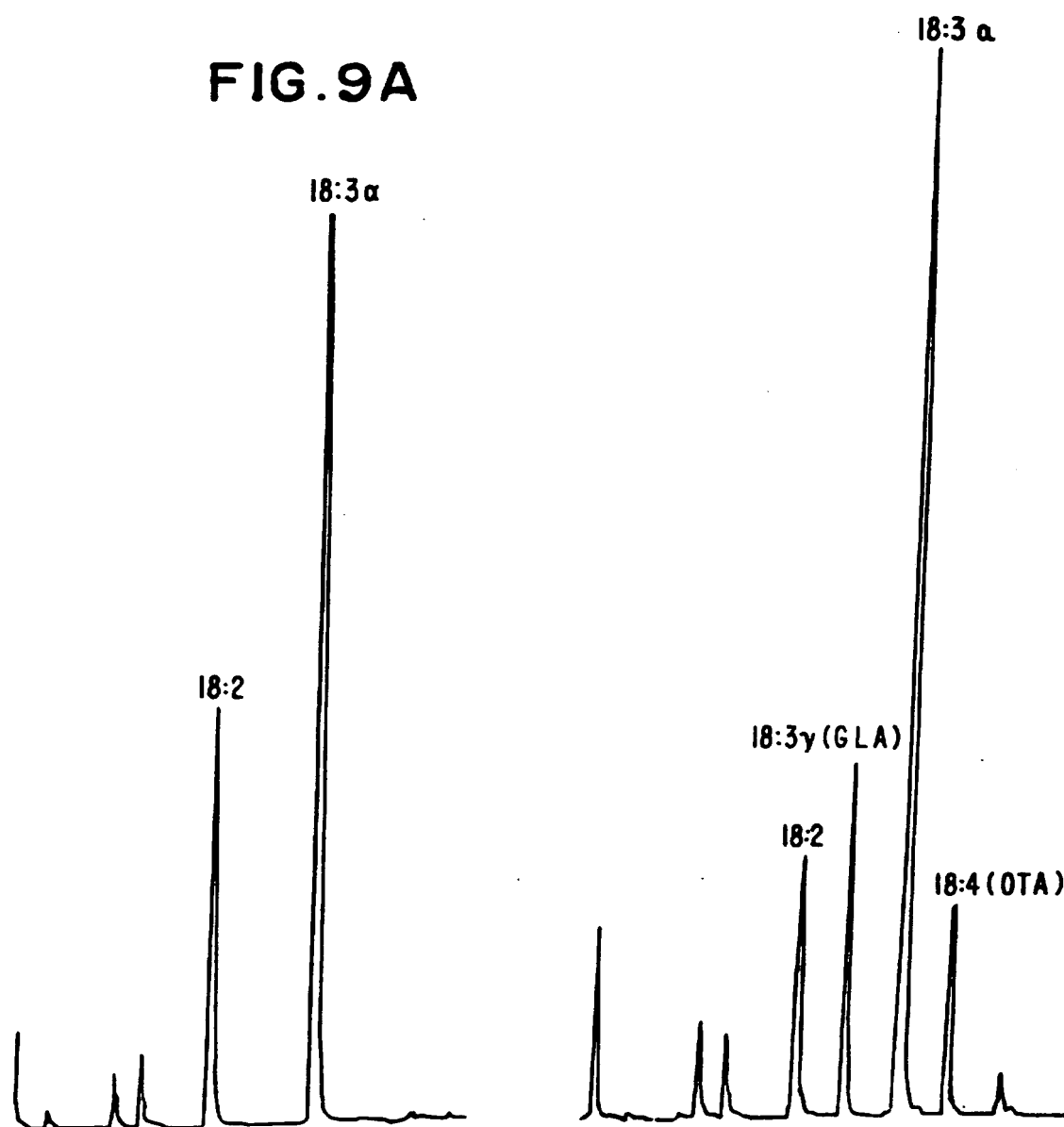


FIG. 9B



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FIG. 10A

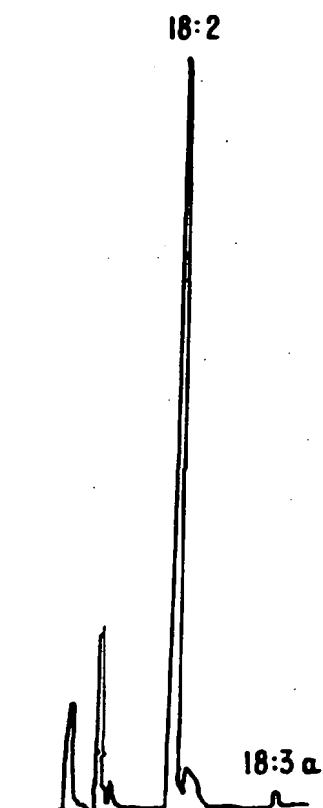
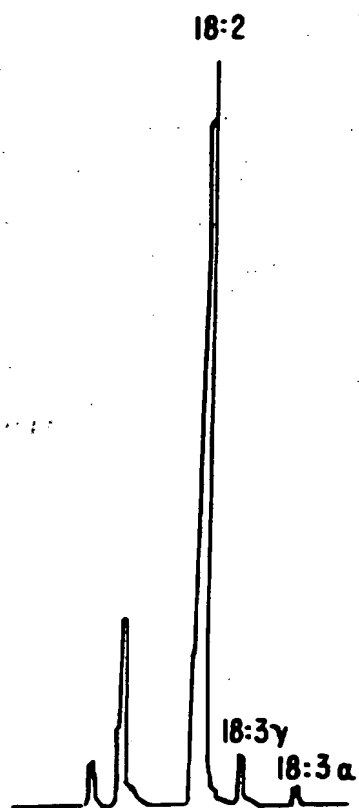


FIG. 10B



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